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# Sex Differences in Uropathogenic Escherichia coli Urinary Tract Infection

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Sex Differences in Uropathogenic *Escherichia coli* Urinary Tract Infection

by

Patrick David Olson

A dissertation presented to  
The Graduate School  
of Washington University in  
partial fulfillment of the  
requirements for the degree  
of Doctor of Philosophy

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## TABLE OF CONTENTS

List of Figures .....	v
Acknowledgments.....	vii
Abstract .....	ix
<b>Chapter 1: Introduction to the Dissertation.....</b>	<b>1</b>
Sex as a biological variable .....	1
Host factors influencing UTI.....	2
Sex and UTI .....	3
Pathogenesis of bacterial cystitis.....	4
Male UTI .....	7
Hormones and UTI.....	8
Summary .....	9
References .....	12
<b>Chapter 2: Androgens Enhance Male Urinary Tract Infection Severity in a New Murine Model.....</b>	<b>23</b>
Abstract .....	23
Introduction .....	24
Materials and methods .....	26
Results .....	30
Discussion .....	36
Acknowledgements .....	40
Figures.....	41
References .....	60
<b>Chapter 3: Androgen Receptor Activation Enables Ascending <i>Escherichia coli</i> Renal Abscess Formation .....</b>	<b>68</b>
Abstract .....	68
Introduction .....	69
Results .....	71
Discussion .....	79
Materials and methods .....	84
Acknowledgements .....	89
Figures.....	89
References .....	106
<b>Chapter 4: Renal Scar Formation and Chronic Kidney Disease after Antibiotic Treatment of Experimental Pyelonephritis .....</b>	<b>116</b>

Abstract .....	116
Introduction .....	117
Materials and methods .....	119
Results .....	122
Discussion .....	127
Acknowledgements .....	131
Figures .....	132
References .....	143
<b>Chapter 5: Concluding Remarks and Future Directions .....</b>	<b>150</b>
Androgen receptor influence on UTI susceptibility .....	151
UPEC intratubular abscess communities .....	154
Pyelonephritic scarring.....	157
Concluding remarks .....	159
<b>Appendix 1: Escherichia coli in Urinary Tract Infections .....</b>	<b>164</b>
Abstract .....	164
Introduction .....	165
Epithelial Adhesion and Pili.....	167
Toxins.....	168
Other virulence determinants .....	170
Invasion of UPEC into the uroepithelium .....	173
Intracellular lifestyle of UPEC during cystitis .....	174
Chronic cystitis.....	178
Immune response and evasion.....	178
Host genetic susceptibility determinants.....	184
Antimicrobial resistance and anti-virulence strategies.....	186
Vaccine prospects.....	188
Conclusion.....	190
Figures .....	191
References .....	194
<b>Appendix 2: Subversion of Host Innate Immunity by Uropathogenic <i>Escherichia coli</i> ....</b>	<b>217</b>
Abstract .....	217
Introduction .....	218
Immune evasion strategies of UPEC.....	220
Does inflammation ultimately <i>promote</i> UPEC infection? .....	225
Conclusions and Future Directions .....	226
Acknowledgments .....	227
Figures.....	228

References ..... 230

## LIST OF FIGURES

### Chapter 1: Introduction to the Dissertation

### Chapter 2: Androgens Enhance Male Urinary Tract Infection Severity in a New Murine Model

Figure 1. Direct bladder inoculation reliably infects the urinary tract of C57BL/6 mice .....	54
Figure 2. Male and female C57BL/6 hosts exhibit differences in bladder cytokine expression 24 hpi.....	55
Figure 3. Cystitis in male C57BL/6 mice follows the IBC pathway.....	57
Figure 4. UPEC more readily colonize the male C3H/HeN bladder.....	59
Figure 5. Male and female C3H/HeN hosts exhibit modest differences in bladder cytokine expression during acute cystitis.....	61
Figure 6. Male C3H/HeN mice develop chronic cystitis .....	62
Figure 7. UPEC display similar bacterial morphology and luminal colonization in chronically infected male and female C3H/HeN mice.....	63
Figure 8. Female and male mice with chronic cystitis display similar elevations in bladder pro-inflammatory cytokines .....	64
Figure 9. Male C3H/HeN mice display acute inflammatory biomarkers predictive of chronic outcome .....	66
Figure 10. Male C3H/HeN mice develop severe pyelonephritis and 100% penetrant renal abscess .....	67
Figure 11. Male C3H/HeN surgically infected with a urosepsis isolate, CFT073, develop more severe chronic UTI.....	69
Figure 12. Male C3H/HeN develop severe renal abscess and fibrosis .....	70
Figure 13. Testosterone enhances susceptibility to chronic UTI .....	71

### Chapter 3: Androgen Receptor Activation Enables Ascending *Escherichia coli* Renal Abscess Formation

Figure 1. Timeline of renal abscess formation in C3H/HeN mice .....	103
Figure 2. Male C3H/HeN do not have renal parenchymal involvement 3 dpi.....	105
Figure 3. Male C3H/HeN develop microabscess 5 dpi .....	106
Figure 4. UPEC establish biofilm-like communities in a protected intraluminal niche.....	107
Figure 5. Female C3H/HeN are susceptible to UPEC intratubular abscess community formation .....	109
Figure 6. Fibrotic tubular epithelium restricts infection but protects UPEC from infiltrating phagocytes .....	110
Figure 7. Type 1 pili are necessary for intratubular community formation .....	111
Figure 8. UTI89ΔfimH ascend to the kidneys in numbers equivalent to wild-type 1 hpi .....	112
Figure 9. Androgen exposure aggravates UTI severity in females .....	113
Figure 10. Surgical induction of cystitis in male C3H/HeN also induces prostatitis .....	114

Figure 11. Testosterone influence on UTI severity is not mediated via aromatization.....	115
Figure 12. Androgen receptor activation enhances severity of UTI .....	117

#### **Chapter 4: Renal Scar Formation and Chronic Kidney Disease after Antibiotic Treatment of Experimental Pyelonephritis**

Figure 1. Ceftriaxone treatment eliminates renal bacterial burden in male C3H/HeN .....	145
Figure 2. Ceftriaxone treated mice display renal abscess 11 dpi despite resolution of infection.....	147
Figure 3. Ceftriaxone treated mice clear infection and develop gross renal scars by 28 days post-treatment .....	149
Figure 4. Treated mice demonstrate healthy renal function 28 days post treatment.....	151
Figure 5. Pyelonephritic scars demonstrate progressive inflammation.....	153
Figure 6. Ceftriaxone-treated mice do not develop markers of end-stage renal disease at 5 months post-treatment .....	154
Figure 7. Infection resolution leaves residual hydronephrosis 5 months post-treatment .....	155

#### **Chapter 5: Concluding Remarks and Future Directions**

##### **Appendix 1: *Escherichia coli* in Urinary Tract Infections**

Figure 1. The intracellular bacterial community model of bacterial cystitis.....	204
Table 1. Host factors influencing bacterial adherence and invasion .....	206

##### **Appendix 2: Subversion of Host Innate Immunity by Uropathogenic *Escherichia coli***

Figure 1. Intracellular UPEC communities are protected from neutrophil attack.....	241
Figure 2. Filamentous UPEC resist phagocytosis in the bladder .....	242



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Patrick D. Olson

*Washington University in St. Louis*

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Dedicated to Katie.

## ABSTRACT OF THE DISSERTATION

Sex Differences in Uropathogenic *Escherichia coli* Urinary Tract Infection

by

Patrick David Olson

Doctor of Philosophy in Biology and Biomedical Sciences

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Associate Professor David A. Hunstad, Chair

Urinary tract infections (UTIs) are among the most common bacterial infections which plague humans. Community-onset UTI is widely viewed as a disease only of women; indeed, its occurrence between 2 and 60 years of age is almost exclusive to females. However, the disease also affects substantial male patient populations, namely infants and the very elderly. While female cases of complicated and upper-tract UTI (pyelonephritis) outnumber male cases overall, men carry an increased risk of mortality from these infections. Sex differences in UTI epidemiology have heretofore been attributed almost entirely to anatomic and hygienic factors in females, including the permissiveness of surrounding environments to microbial colonization, shorter urethral length, and shorter distance from anus to the urethral meatus. However, these hypotheses and potential mechanisms driving sex differences in UTI have not been investigated due to a lack of preclinical models allowing sex comparisons.

In response to this deficit, we devised a minimally invasive surgical bladder inoculation technique that yields reproducible upper and lower UTI in both male and female mice. Acute uropathogenic *Escherichia coli* (UPEC) infection in males of two mouse strains recapitulated the intracellular bacterial community pathway previously shown in females. Compared with females,

C3H/HeN males displayed a striking predilection for chronic cystitis, manifesting as persistent bacteriuria, high-titer bladder bacterial burdens, and chronic inflammation as previously characterized in catheter-infected C3H/HeN females. Further, males developed more severe pyelonephritis, as well as nearly 100% penetrant renal abscesses, a complication that is rare in female mice. Within the abscess, UPEC formed a dense nidus of intratubular, biofilm-like communities that appeared sheltered from surrounding inflammatory cells by a fibrotic capsule. The formation of these abscess communities required type 1 pili, a major urovirulence determinant in the bladder but not previously implicated in pyelonephritis.

Severe ascending UTI in males was sharply abrogated following castration, but complemented with exogenous testosterone. Similarly, administration of exogenous testosterone in C3H/HeN females imparted susceptibility to severe pyelonephritis and abscess formation. Susceptibility to severe UTI was also induced in both sexes with 5- $\alpha$ -dihydrotestosterone or attenuated by treatment with the antiandrogen flutamide. Further, male mice lacking a functional androgen receptor were protected from severe upper-tract UTI. Collectively, these data show that specific activation of the androgen receptor augments severity of UTI in both sexes.

Our mini-surgical model provides a new preclinical platform for translatable studies of UTI pathogenesis in both male and female hosts, specifically expanding studies of pyelonephritis, ascending renal abscess development, and the treatment of complicated UTI. The discovery of intratubular UPEC communities in this model offers a first look at early renal abscess pathogenesis and helps to explain the need for prolonged antimicrobial treatment during severe pyelonephritis. Further, our findings suggest that modulation of androgens may represent a novel therapeutic route to combat recalcitrant or recurrent UTI, and may help to explain why certain patient populations are more likely to develop UTI.

# CHAPTER ONE

## INTRODUCTION TO THE DISSERTATION

Modified from Olson and Hunstad (2016) Sex and other factors influencing urinary tract infection pathogenesis. In: Bjerklund Johansen TE, Wagenlehner FME, Cho YH, Matsumoto T, Krieger JN, Shoskes D, Naber KG, eds. *Urogenital Infection and Inflammation*. Arnhem, Netherlands: European Association of Urology (in press).

### **Sex as a biological variable**

“From womb to tomb” *it matters*, argues the 2001 Institute of Medicine report (1); biological sex should be a fundamental consideration in human health and disease. Indeed, a number of human diseases manifest profound sex-based differences in prevalence, incidence, severity, and response to treatment. Yet, sex as a biological variable has long been ignored experimentally in the biomedical sciences and clinically in the application of evidence-based medicine (2, 3). Research and medicine in the US and other countries are currently on the verge of a paradigm shift in which sex should and will increasingly be considered from bench to bedside (2-4).

Such a sex bias is well entrenched in our understanding of urinary tract infections (UTIs), a collection of diseases which have been both prejudiced and understudied because of their disproportionate affliction of women and long-standing experimental and clinical sex biases. UTIs are among the most common bacterial infections that plague humans. On an epidemiologic basis, community-onset UTI is widely viewed as a disease only of women; indeed, its occurrence between 2 and 60 years of age is almost exclusive to females, and it is estimated that at least half of American women will suffer a UTI during their lifetimes (5). However, in certain populations the incidence of male UTI matches or exceeds that in females; epidemiologic data also suggest a sex difference in morbidity from upper-tract UTI (6-9). To better understand resistance and

susceptibility mechanisms to UTI, it is important that we consider age- and sex-related discrepancies that are evident in the disease, and how sex and other host factors may influence the pathogenesis and outcomes of ascending UTI.

### **Host factors influencing UTI**

Though contemporary and emerging technologies will enable further investigation of the presumed sterility of the urinary tract, microbiologic health of the urinary tract depends on multiple host factors, including a finely tuned innate inflammatory response, which act to eliminate potential uropathogens from the bladder and kidneys (10-13). Despite repetitive exposure of this potentially hospitable, nutrient-rich environment to bacterial pathogens (*e.g.*, following sexual intercourse (14)), bacteriuria in the otherwise healthy human host usually is transient (15-17). Host traits that compromise the defenses of the urinary tract augment disease susceptibility, severity, and progression. Forward flow of urine provides a formidable mechanical defense; dysfunctional voiding and other urodynamic abnormalities are clearly associated with increased susceptibility to UTI in children and adults (18). Such conditions in the adult female likely include pregnancy, which also predisposes to UTI (19). Factors which increase the hospitable of the bladder microenvironment to infection, such as glycosuria associated with diabetes mellitus, likewise increase susceptibility to UTI (20); hyperglycemia may also additionally compromise the activity of phagocytes. Finally, impairment of the innate immune response either by immunosuppression or genetic defects in innate components lead to increased frequency of UTI (12). Multiple efforts to define human genetic variation imparting susceptibility to UTI have been modestly successful. Attention in this realm has been focused largely on host innate immune genes. However, it has also become clear that susceptibility is

both polygenic and environmental, and that genetic determinants of distinct clinical syndromes (asymptomatic bacteriuria, cystitis, and pyelonephritis) must be pursued independently.

## **Sex and UTI**

However, the host trait that is most influential in the development of UTI is undeniably biological sex. The frequency of UTI changes drastically across the lifespan and varies by sex (21). As mentioned previously, the occurrence of UTI in middle age is almost exclusive to females. However, certain populations of males show an increased risk of UTI. The sex discrepancies seen in UTI epidemiology have been traditionally attributed to anatomic (and, less conclusively, hygienic) factors, including the permissiveness of the surrounding vaginal and perineal environments to microbial colonization, a shorter distance from the anus to the urethral meatus, and shorter urethral length in females.

UTI in young children is common (22, 23). The sex ratio in UTI incidence among infants is approximately 2:1 – still favoring females, but at a much lower ratio than in later childhood and beyond. In fact, many studies have shown that male UTI cases outnumber females within the first 3-6 months after birth (24-31). Thereafter, UTI susceptibility wanes in males through later infancy. Infants and young children thus may represent a unique patient population in which to investigate sex differences in UTI. Clinically, prompt diagnosis and treatment of UTI in infancy is necessary to prevent renal scarring and potential long-term complications (32). Interestingly, a history of maternal UTI during pregnancy has been associated with up to a 5.9-fold higher risk of UTI in both sexes during infancy (33, 34). It is unclear if this risk arises from inheritance of genetic predisposition to UTI, intrapartum or postpartum transmission of virulent uropathogens

from the maternal genitourinary tract to the microbiota of the infant, or other environmental factors.

Though women represent >90% of UTI patients between early childhood and late middle age (5, 35, 36), complicated UTI manifests in both sexes across this time frame in patients with indwelling urinary catheters, diabetes mellitus, spinal cord injuries, immunosuppression, and structural or urodynamic abnormalities. Notably, while female cases of complicated and upper-tract UTI (pyelonephritis) outnumber male cases overall, men carry an increased risk of mortality from these infections (6-9). Thousands of men, particularly those of advancing age, also suffer from acute and chronic bacterial prostatitis, a clinical diagnosis with signs, symptoms, and etiological pathogen profiles that overlap substantially with those of lower UTI in females (37, 38). The incidence of UTI in males increases substantially after 60 years of age, largely because of abnormal voiding patterns due to acquired urodynamic abnormalities (*e.g.*, benign prostatic hyperplasia) (5, 21, 39). In total, UTIs comprise debilitating diseases with substantial morbidity and occasional mortality among males (37, 38).

### **Pathogenesis of bacterial cystitis**

Though a number of distinct bacterial pathogens may cause UTI and prostatitis, uropathogenic *Escherichia coli* (UPEC) predominate among etiological agents in both sexes, causing >80% of community-onset UTI, roughly one fourth of hospital-acquired UTI, and >70% of infectious prostatitis (5, 21, 40). Our knowledge of the molecular details of UPEC pathogenesis has been developed largely in an exclusively female murine model. In this widely used model of bacterial cystitis, female mice are briefly catheterized and uropathogenic bacteria inoculated into the bladder lumen; however, the bladders of male mice are not reliably accessed



by catheter, precluding studies of male cystitis and pyelonephritis using this method. Upon delivery to the female bladder, UPEC and other uropathogens first encounter the stratified transitional epithelium, lined by a single layer of large, multinucleated superficial facet cells apically coated with an array of integral membrane proteins known as uroplakins (41). UPEC exploit mannose moieties decorating these uroplakins as the receptor for their major virulence determinant, adhesive type 1 pili (42, 43). UPEC genomes typically contain multiple discrete loci encoding highly resilient, heteropolymeric pili that are assembled via the canonical chaperone-usher secretion pathway (44). Absence of type 1 pili or the tip adhesin FimH abolishes bacterial attachment and subsequent cystitis (45, 46). In a similar way, P pili can bind globoseries glycolipids found on the renal epithelium (47, 48), potentially accounting for the predilection of P-piliated *E. coli* to adhere to kidney tissue and cause pyelonephritis (49).

Following type 1 pilus-mediated attachment to the uroepithelium, UPEC are rapidly internalized into superficial facet cells (45, 46, 50-65). The FimH adhesin is both necessary and sufficient for uroepithelial invasion (51, 60, 66). After a single bacterium has invaded a facet cell, it may then rapidly replicate in the host cell cytoplasm, initially forming loose collections that subsequently coalesce into densely packed intracellular bacterial communities (IBCs) (55). Infected bladder epithelial cells may be exfoliated, eliminating some IBCs in the urine (67, 68); meanwhile, a subset of UPEC may flux from the mature IBC, assume a filamentous morphology that resists neutrophil phagocytosis, and initiate further rounds of IBC formation by invading nearby naive epithelial cells (69, 70). Murine and *in vitro* evidence for this IBC cascade has been corroborated by detection of shed IBCs in female human urines (71, 72), suggesting that the murine IBC pathway recapitulates acute cystitis in women.

There are two phenotypic outcomes of murine cystitis, which are dependent on the host genetic background used. Following resolution of acute infection in typical model hosts (*e.g.*, C57BL/6 females), UPEC may establish a quiescent intracellular reservoir (QIR) within the bladder epithelium that resists systemic antibiotic therapy and is also protected from host immune mechanisms (45, 73). Such a reservoir can persist for months, during which the urine is sterile, with occasional “spikes” of cultivatable *E. coli*. However, UPEC are readily recovered from bladder tissue up to 4 months later (74), and there is no histologic evidence of inflammation in these chronically colonized tissues (45). Consequently, QIRs are believed to represent seeds for same-strain recurrent UTI. In susceptible murine hosts (*e.g.*, C3H/HeN), a fraction of mice are unable to resolve acute UTI, developing long-term chronic bacterial cystitis with ongoing high tissue bacterial burden and inflammation (75). By 2 weeks post-infection (wpi), C3H/HeN females show a bimodal phenotype, with an inoculum-dependent fraction of mice developing chronic cystitis while remaining mice resolve infection to the QIR state. The nature and amplitude of the immune response within the first 24 hours of infection may influence the fate of individual C3H females (75).

While our knowledge of the UPEC intracellular cascade and murine infection outcomes has increased exponentially, studies have been completed entirely in females. Thus, it has been unclear how the IBC pathogenic cascade proceeds during male cystitis and if there are differences in murine infection outcome. Likewise, we know relatively little about the pathogenic mechanisms for ascending bacterial infection of the kidney in comparison to our understanding of the intracellular cascade used during bacterial cystitis. Female mice are largely resistant to ascending pyelonephritis in the catheterization model (75). Consequently, our knowledge of the virulence repertoire required by uropathogens, mechanisms for bacterial

persistence and propagation, and host response to infection within the kidney are incompletely defined.

## **Male UTI**

Unfortunately, the epidemiology, diagnosis, and treatment of UTI in male populations have been poorly described compared to the robustly studied female populations, although substantial differences exist between them (76). Infections of male accessory organs, including prostatitis, epididymitis, orchitis, and seminal vesiculitis, can also be classified as exclusively male forms of UTI. UTI in adult male patients is often viewed as “complicated,” because these organs are sometimes involved and due to the relative paucity of cases in men compared to women.

Males also exhibit an increase in some specific risk factors that potentiate UTI. A lack of circumcision increases risk for UTI in both infants (77-79) and adult men (80). Men have a striking predilection for spinal cord injury, outnumbering female cases four to one; chronic, recurrent UTIs present a difficult and often lifelong challenge in patients with neurogenic bladder arising from such injuries and other causes (81). Complicated UTI also manifests in hospitalized males with indwelling urinary catheters, those receiving immunosuppression, and those with structural abnormalities (particularly in infancy).

Optimal treatment regimens (antibiotic choice and duration) for uncomplicated UTI in females have been the subject of numerous clinical trials, and the contemporary clinician can rely on published guidelines for these patients (82), while evidence supporting proper choice of antimicrobial agents and duration of therapy for men is less clear. Many expert recommendations call for extended (14 days or longer) courses of antibiotic therapy to treat male

UTI (39, 83-85). However, a recent study found no differences in acute resolution or recurrence rate between male UTI patients treated for < 7 days versus those treated for > 7 days; moreover, longer-duration therapy was associated with the subsequent development of *Clostridium difficile* infection (86). Ongoing studies and clinical trials are expected to inform the development of more evidence-based and specific recommendations for treating male UTI (87).

## **Hormones and UTI**

Developing evidence suggests that hormonal milieu may impact UTI susceptibility and severity. Our increasing knowledge of this field is particularly interesting as it may ultimately inform approaches that represent alternatives to antibiotic treatment.

There are extensive but somewhat conflicting data on the influence of estrogen on susceptibility to UTI. Young adult women, who exhibit the highest estrogen levels, account for the majority of community-onset UTI cases, and high estrogen levels have been linked to increased UTI susceptibility (88). However, post-menopausal females also experience an increased incidence of UTI, which in some cases has been treated with estrogen supplementation. Results from murine studies employing ovariectomized and/or estrogen-supplemented females to examine the influences of estradiol on UTI pathogenesis are likewise conflicting. Some studies have found modest increases in bladder bacterial burdens in estrogen-depleted hosts, particularly during the acute phase of cystitis (89, 90). Conversely, Curran *et al.* noted an increased risk of upper-tract UTI in mice following estrogen treatment (91). These experimental models notably bypass the vaginal and periurethral microenvironment, as mice are inoculated by transurethral catheterization of the bladder. Collectively, the available data suggest that the impact of estrogen on bacterial pathogenesis within the urinary tract proper is

likely minor. However, many have posited that estrogens may influence periurethral colonization by uropathogens and alter UTI-relevant facets of the vaginal environment (*e.g.*, composition of the vaginal microbiome, dryness, sexual intercourse frequency). In line with this hypothesis, a Cochrane review concluded that topical estrogens show possible benefit in reducing UTI risk, albeit with a number of side effects (92). Of note, recent evidence suggests that high estradiol levels may cause opposing changes in both bladder fortification and receptivity to infection, providing a viable hypothesis for inconsistent estrogenic influences on UTI. High estradiol levels may induce expression of major adhesive receptors for uropathogens in bladder epithelial cells *in vitro* (thereby promoting increased bacterial adherence and invasion), while a protective effect may be attributable to its ability to induce antimicrobial peptides during acute UTI (90). The influence of other hormones in the hypothalamic-pituitary-gonadal axis, including testosterone, on UTI susceptibility has not been explored.

## **Summary**

Men and women display fundamental differences in their susceptibility to infectious diseases. These dissimilarities may stem from a multitude of differences: in pathogen exposure, cultural and behavioral issues, anatomy, hormonal expression, treatment efficacy, socio-economic influences, and many more. Most notably, sex differences in immunity have been clearly described, with females displaying enhanced resistance to many infections because of more robust immune responses controlling pathogens (93-95). This enhanced defense in women has been hypothesized to have evolved from the need to protect their fetuses from infection (96), and is associated with the greater frequency and severity in women of many chronic inflammatory and autoimmune diseases (97, 98).

Ascending UTI represents a contradiction to this paradigm, being one of the few infectious diseases which disproportionately afflicts females over males. In considering how biological sex influences UTI, the hypothesized protective mechanism for males is anatomy, while repeated introduction of bacterial pathogens to the urinary tract in females necessitates a finely tuned innate surveillance and defense system as primary protection. Traits or interventions which bypass or hamper these defenses in females represent risk factors for chronic and/or recurrent UTI (13). Sex differences in UTI epidemiology have heretofore been attributed almost entirely to anatomic and hygienic factors, including the permissiveness of vaginal and perineal environments to microbial colonization, shorter urethral length, and shorter distance from anus to the urethral meatus in females (14, 21, 99). However, these hypotheses have not been stringently proven, and substantial deficiencies persist in our understanding of the interplay between host sex and uropathogens.

There have been recent calls by the US National Institutes of Health (4) and in the basic and clinical literature (2, 100, 101) for sex-based research approaches to infectious and other diseases, including a specific focus on UTI (86, 102). A greater understanding of how biological sex influences UTI promises to inform the development of novel therapeutics and interventions, yielding better sex-based treatment and prevention strategies for the benefit of all patients.

Substantial data from the current murine model and female patients suggest that the IBC represents a protected replicative niche, necessary for the establishment and progression of UTI in female hosts. However, this emerging paradigm has not been extended to male hosts, and sex influences on the intracellular pathogenic cascade may underlie the observed sex discrepancies in UTI in human patients. Technical barriers to infecting male mice have heretofore precluded the extensive study of male UTI in model hosts, resulting in a tremendous knowledge gap regarding

host and microbial mechanisms underlying sex discrepancies in this disease. This body of work sought to address this gap by developing an innovative minimally-invasive surgical technique that bypasses simple anatomic differences to permit infection of the urinary tract in male and female model hosts. This significant advance allowed us to explore the central hypothesis that intrinsic sex differences in epithelial receptivity and/or immune responses of the urinary tract underlie phenotypic sex differences in susceptibility to these infections. The new model also emerged as a novel platform for modeling the pathogenesis and sequelae of severe pyelonephritis with ascending abscess formation in male mice.

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CHAPTER TWO

ANDROGENS ENHANCE MALE URINARY TRACT INFECTION SEVERITY IN A NEW  
MURINE MODEL

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**Abstract**

Urinary tract infections (UTIs) occur predominantly in females but also affect substantial male patient populations; indeed, morbidity in complicated UTI is higher in men. Because of technical obstacles, preclinical modeling of UTI in male mice has been limited. We devised a mini-surgical bladder inoculation technique that yields reproducible upper and lower UTI in both male and female mice, enabling studies of sex differences in these infections. Acute

uropathogenic *Escherichia coli* cystitis in males of two mouse strains recapitulated the intracellular bacterial community pathway previously shown in females. However, surgically infected C57BL/6 and C3H/HeN females exhibited more robust bladder cytokine responses and more efficient UPEC control than males. Compared with females, C3H/HeN males displayed a striking predilection for chronic cystitis, manifesting as persistent bacteriuria, high titer bladder bacterial burdens, and chronic inflammation. Further, males developed more severe pyelonephritis, as well as 100% penetrant renal abscess (a complication that is rare in female mice). These phenotypes were sharply abrogated following castration, but complemented with exogenous testosterone, suggesting that male susceptibility to UTI is strongly influenced by androgen exposure. These data substantiate the long-standing presumption that anatomic differences in urogenital anatomy confer protection from UTI in men; however, as observed clinically, male sex was associated with more severe UTI once these traditional anatomic barriers were bypassed. This study introduces a highly tractable preclinical model for interrogating sex differences in UTI susceptibility and pathogenesis, and illuminates a more complex interplay between host sex and UTI than previously appreciated.

## **Introduction**

Community-onset urinary tract infection (UTI) disproportionately afflicts women (1); however, routine and complicated UTIs in male patients are neither nonexistent nor benign. Males at the extremes of age, namely infants and the very elderly, exhibit increased incidence of UTI. Further, complicated UTI commonly manifests in males with urodynamic problems (e.g., spinal cord injury, neurogenic bladder), indwelling urinary catheters, immunosuppression, and/or structural abnormalities. Importantly, while female cases of complicated and upper-tract UTI

(pyelonephritis) outnumber male cases overall, men carry an increased risk of mortality from these infections (2-5). Millions of men also suffer from acute and chronic bacterial prostatitis. In total, UTIs comprise debilitating diseases with substantial morbidity and appreciable mortality specific to males (6, 7). Of note, uropathogenic *Escherichia coli* (UPEC) is the predominant cause of UTI in both sexes, causing >80% of community-onset UTI, ~25% of hospital-acquired UTI, and >70% of infectious prostatitis (1, 8-10).

Sex differences in UTI epidemiology have heretofore been attributed almost entirely to anatomic and hygienic factors, including the permissiveness of vaginal and perineal environments to microbial colonization, shorter urethral length, and shorter distance from anus to the urethral meatus in females (9, 11, 12). However, these hypotheses have not been stringently proven, and substantial deficiencies persist in our understanding of the interplay between host sex and uropathogens. Recent advances in our translational view of UTIs and in the molecular details of UPEC pathogenesis have been developed in an exclusively female murine model where pathogens are delivered into the bladder lumen by transurethral catheterization (13, 14). however, the bladders of male mice cannot be reliably catheterized. Upon delivery to the female bladder, UPEC are rapidly internalized into superficial facet cells lining the stratified transitional epithelium (15-26), subsequently replicating in the cytoplasm to form biofilm-like masses of bacteria termed intracellular bacterial communities (IBCs) (21). Murine evidence for this IBC cascade has been corroborated by detection of exfoliated IBCs in the urine of women and girls with cystitis (27, 28). Female mice either resolve acute cystitis or develop chronic bacterial cystitis, while very few develop lasting kidney infection or renal abscess (29, 30). While chronic cystitis as described in murine hosts may not be common clinically with routine antibiotic

treatment, increasing evidence suggests this to be a relevant model for severe and recurrent UTI (31, 32).

Previous studies have instilled uropathogens into the urethra of male mice (33, 34), inducing infection of the prostate but failing to reproducibly infect the bladders and kidneys. The lack of a tractable male model of UTI has precluded detailed examination of host and microbial mechanisms underlying the sex discrepancies observed in UTI. In response to this deficit, we developed a mini-surgical technique that bypasses simple anatomic sex differences to permit infection of the urinary tract in both male and female mice. Acute cystitis utilized an IBC pathway in males which mirrored acute bladder infection in females. While most surgically infected female mice controlled UPEC effectively, males exhibited a sharply higher prevalence of chronic cystitis. More striking, male mice exhibited marked susceptibility to renal abscess and pyelonephritis, mirroring the increased morbidity reported in men who develop these complicated UTIs. These male UTI phenotypes were reversed by gonadectomy but complemented via exogenous testosterone administration, implicating androgens in susceptibility to severe UTI. Collectively, this study illuminates sex-based influences on susceptibility and host response to UTI and establishes a novel platform for modeling the pathogenesis and sequelae of cystitis, pyelonephritis, and ascending renal abscesses.

## **Materials and methods**

### *Bacterial strains and growth conditions.*

Uropathogenic *E. coli* strain UTI89 was isolated from a patient with cystitis (35). UPEC strain CFT073 was isolated from the blood of a patient with acute pyelonephritis (36). For surgical infections, bacteria were grown statically in Luria-Bertani (LB) broth for 16 h at 37°C to

induce type 1 piliation. The cultures were centrifuged at  $7,500 \times g$  for 10 min at  $4^{\circ}\text{C}$  before resuspension in sterile phosphate-buffered saline (PBS) to a final density of  $\sim 4 \times 10^8$  CFU/ml.

#### *Surgical murine model of UTI.*

Animal care and use protocols received prior approval from the Washington University Animal Studies Committee. Experiments were conducted in inbred C57BL/6 (Jackson Laboratories, Bar Harbor, ME) or C3H/HeN (Harlan Laboratories, Indianapolis, IN) murine backgrounds. Mice were housed under constant temperature and humidity, with a 12-h light/dark cycle. The female murine model of cystitis with transurethral inoculation via catheter has been described in methodologic detail (13, 37). For mini-surgical infection, mice aged 8-9 weeks were maintained under inhalation anesthesia with 3% isoflurane via vaporizer and nose cone. Anesthetized mice were positioned supine, and the ventral abdomen was shaved and disinfected with 70% alcohol and 2% chlorhexidine solution. A 2-3 mm, vertical, midline incision was made directly overlying the bladder, first through the abdominal skin and then the peritoneum. The bladder was exposed through the incision using gentle bilateral pressure on the abdomen and aseptically emptied. The inoculum was prepared in a 1-mL tuberculin syringe adapted to a 30-gauge, 0.5-inch needle. The needle was introduced to the bladder lumen near the neck of the bladder at a 45-degree angle. The lumen was inoculated with 50  $\mu\text{L}$  of bacterial suspension (containing  $1-2 \times 10^7$  CFU) over a period of 10 s. The bladder was allowed to expand for an additional 10 s before withdrawing the needle; the injection site sealed immediately with no evident leakage. The bladder was gently replaced, the peritoneum and skin were closed separately with simple, interrupted sutures, and the animal was awakened in fresh air. Infections were allowed to proceed for durations from 6 h to 4 weeks.

#### *Determination of urine and tissue bacterial loads.*

Post-infection clean-catch urine samples were collected using gentle suprapubic pressure, serially diluted and plated to LB agar to enumerate CFU. For organ titers, mice were euthanized by CO<sub>2</sub> asphyxiation, and bladders and kidney pairs were aseptically removed, homogenized in 1 ml or 0.8 ml (respectively) of sterile PBS, and serially diluted and plated. Cystitis in C3H/HeN mice was termed “chronic” if endpoint bladder and all urine titers were  $>10^4$  CFU/ml, and “resolved” if endpoint bladder burdens or at least one urine titer were  $<10^4$  CFU/ml (29, 30, 32).

#### *Tissue and serum cytokine analysis.*

The concentrations of 23 mouse cytokines in whole-bladder homogenates or sera were analyzed with a multiplex bead array platform (Bio-Plex; Bio-Rad, Hercules, CA) as described previously (29, 38). Data represent the mean and standard deviation from five individual mice across 2 biological replicates per time point, each analyzed in technical duplicate.

#### *IBC enumeration and confocal microscopy.*

For IBC quantification, bladders were bisected, splayed, fixed, and stained for bacterial  $\beta$ -galactosidase as previously described (39). IBCs were counted under a dissecting light microscope. For IBC confocal microscopy, splayed bladders of C57BL/6 male and female mice were examined at 16 hpi with GFP-expressing UTI89, as described previously (39). Bacteria present at chronic time points were visualized by immunofluorescence. Splayed bladder halves were fixed in 2.5% paraformaldehyde, blocked in 1% BSA, 0.3% Triton X-100 in PBS for 1 h, incubated with rabbit anti-*E. coli* (E3500-06C, US Biological, Salem, MA) and secondary



AlexaFluor 488-conjugated goat anti-rabbit IgG (Life Technologies, Grand Island, NY) antibodies, and stained with SYTO 61 red fluorescent nucleic acid stain (Molecular Probes, Eugene, OR). Images were acquired on a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss Inc., Thornwood, NY).

*Ex vivo gentamicin protection assay.*

Quantification of intracellular and extracellular bacteria in the murine bladder 6 hpi was performed by a modified *ex vivo* gentamicin protection assay, as previously described (40). Bladders were aseptically harvested at 6 hpi, bisected, and washed vigorously in PBS, which was plated to enumerate extracellular bacteria. Following 90 min incubation at 37°C in PBS containing gentamicin (100 µg/ml), bladders were washed with PBS, then homogenized and plated for intracellular CFU.

*Bladder and kidney histopathology.*

Bladder and kidneys were bisected and fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid). Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Gomori trichrome stain.

*Serum collection and storage.*

Venous blood was collected by submandibular puncture using 5-mm steel lancets (MediPoint, Mineola, NY) into Microtainer serum separation tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were allowed to clot for 1 h and were clarified by centrifugation at  $15,000 \times g$  for 5 min before storage at -80°C.

### *Gonadectomy and testosterone implantation.*

Bilateral orchiectomy (castration), bilateral ovariectomy, or sham operations were performed at Harlan Laboratories (Indianapolis, IN) following standard procedures at 5 weeks of age. Animals were allowed to recover 1 week before shipment. Where indicated, 60-day continuous release pellets containing 25 mg testosterone or placebo (Innovative Research of America, Sarasota, FL) were implanted subcutaneously at the nape of the neck 5 days following castration or sham operation. Mice were surgically infected at 9 weeks of age (i.e., 4 weeks following gonadectomy or sham surgery).

### *Statistics.*

Statistical tests for significance were performed using Prism 6 software (GraphPad, La Jolla, CA). Observed differences in bacterial CFU, IBC numbers, and cytokine levels were analyzed with the unpaired, two-tailed, nonparametric Mann-Whitney *U* test. To compare proportions of mice developing persistent bacteriuria and chronic cystitis or renal abscess, a two-tailed Fisher's Exact test was used. *P* values < 0.05 were deemed significant.

## **Results**

### *Direct bladder inoculation reliably infects the urinary tract of C57BL/6 mice.*

We developed a highly reproducible, minimally invasive surgical approach that allows direct male-female comparisons in UTI pathogenesis. We first employed our mini-surgical inoculation model to induce UTI in C57BL/6 male and female mice with the cystitis-derived

UPEC strain UTI89, monitoring bladder and kidney colonization at three time points representing acute and chronic stages of UTI (29, 30, 41) (**Figure 1**). Organ titers in surgically infected female C57BL/6 were similar to published bacterial burdens in catheter-infected females (22, 29, 42-44), indicating that our mini-surgical method recapitulates the widely used female model (13). Mice displayed no signs of systemic infection or peritonitis, and blood cultures were sterile 6 and 24 hours post infection (hpi).

Bladder bacterial burdens in C57BL/6 males and females were equivalent at all time points examined (**Figure 1A**). However, C57BL/6 males developed significantly higher kidney titers 24 hpi ( $P=0.0038$ ) and 2 wpi ( $P=0.0028$ ) compared to females (**Figure 1B**). Male and female C57BL/6 mice expressed similar levels of bladder cytokines and chemokines 6 hpi. By 24 hpi, females displayed modest but statistically significant increases in bladder IL-1 $\alpha$ , IL-10, IL-12 (p40), G-CSF, MIP-1 $\alpha$ , and TNF- $\alpha$  content relative to males (**Figure 2**).

*Cystitis in male C57BL/6 mice follows the IBC pathway.*

The importance of the IBC pathway in cystitis has not been interrogated in male hosts. By LacZ staining (39), IBCs were observed in all infected bladders of both sexes, with no significant differences in IBC number between males and females 6 or 24 hpi (**Figure 3A**). Confocal microscopy revealed no differences in bacterial morphology, size, or structure in early or mature IBCs between sexes (**Figure 3B**). Thus, acute cystitis in male hosts proceeds through an IBC pathway analogous to that previously observed in females.

*UPEC more effectively colonize the male C3H/HeN bladder.*

We next applied our mini-surgical method in C3H/HeN mice (a host strain in which a minority of infected females develop chronic cystitis (29)). Surgically infected male C3H/HeN developed robust acute cystitis, with bladder bacterial loads significantly higher than those in females 6 hpi ( $P=0.0003$ ; **Figure 4A**). Using an *ex vivo* gentamicin protection assay (40), we found modest increases in both the intracellular and extracellular bladder compartments of C3H/HeN males compared to females (**Figure 4B**). As in the C57BL/6 background, we observed no differences between sexes in IBC numbers in C3H/HeN hosts 6 or 24 hpi (**Figure 4C**). We found only modest sex differences in bladder cytokine content during acute cystitis in the C3H/HeN background (**Figure 5**).

*Male C3H/HeN mice are highly susceptible to chronic cystitis.*

In catheter-infected C3H/HeN females, a bimodal distribution of bladder UPEC titers develops by 2-4 wpi; a minority (20-40%) exhibit chronic cystitis while most females resolve bacteriuria (29, 39). As previously defined, chronic cystitis comprises persistent, high-titer bacteriuria ( $>10^4$  CFU/mL), high bladder bacterial load 2-4 wpi, ongoing robust inflammation, and persistent inability of the bladder to regenerate the terminally differentiated superficial facet cell layer (29, 30). Elevation in serum IL-5, IL-6, G-CSF, and KC (CXCL1) 24 hpi constitutes a biomarker signature that predicts development of chronic cystitis in female mice (29).

C3H/HeN females surgically infected with  $10^7$  CFU UTI89 exhibited a phenotypic split as seen in the catheterization model, with 20% of females showing high bladder CFU 2 wpi

(**Figure 4A**). Strikingly, male bladders demonstrated significantly higher bacterial burdens than females 2 wpi ( $P<0.001$ ; **Figure 4A**), with 100% of males developing the high titers typical of chronic cystitis ( $P<0.0001$  vs. female proportion).

To confirm that these high bacterial loads in male bladders indeed reflected the chronic cystitis phenotype, we surgically infected male and female C3H/HeN mice and enumerated bacterial CFU in weekly urine samples and bladder homogenates 4 wpi. Consistent with prior studies (29, 39, 45) and our initial 2-wpi data (**Figure 4A**), a minority (24%) of C3H/HeN females exhibited persistent bacteriuria 4 wpi, compared with 76% of males ( $P=0.0053$  vs. female proportion; **Figures 6A and B**). Histopathological analysis of bladders from female or male mice with persistent bacteriuria demonstrated a hyperplastic transitional cell layer with complete exfoliation of superficial facet cells, severe edema, and chronic, follicle-like inflammatory infiltrates in the submucosa (**Figures 6C and D**). Bladders from male or female mice that resolved bacteriuria lacked any notable pathology 4 wpi (data not shown). Bladders of male and female mice with persistent bacteriuria 2 wpi displayed identical bacterial morphology and luminal colonization by immunofluorescence and confocal microscopy (**Figure 7**). Both male and female C3H/HeN with chronic cystitis showed local elevations in multiple pro-inflammatory cytokines, compared with mice that resolved acute infection (**Figure 8**). Taken together, these data argue that the phenotype observed in high-titer male bladders indeed reflects chronic cystitis as observed in females (29), although male sex is associated with an increased frequency of developing chronic cystitis.

Surgically infected females that developed chronic cystitis demonstrated inflammatory cytokine signatures 24 hpi (i.e., elevated IL-5, IL-6, G-CSF, and KC) comparable to those reported in catheter-infected females (29) (**Figure 9**). Males demonstrating persistent bacteriuria

had significantly higher serum IL-6 24 hpi compared with resolved males ( $P=0.0316$ ); increases in serum IL-5, G-CSF, and KC trended similarly (**Figure 9**). These findings suggest that male C3H/HeN hosts, like females, feature an acute inflammatory checkpoint that influences chronic infection outcome.

*Male C3H/HeN mice develop severe pyelonephritis and renal abscess.*

We hypothesized that the amplified renal infection observed in C57BL/6 males (**Figure 1B**) would be recapitulated more strongly in the C3H/HeN background, a host with increased propensity for vesicoureteral reflux (43, 46). Indeed, surgically infected male C3H/HeN displayed significantly higher renal bacterial loads compared to females ( $P<0.0001$ ; **Figure 10A**). Moreover, while females largely resolved kidney infection over time, renal bacterial loads in males rose substantially from 6 hpi to 2 wpi, with no males able to resolve kidney infection (**Figure 10A**). These excessive bacterial loads in males correlated with grossly evident renal abscess 2 wpi (**Figure 10B**); 100% of males demonstrated gross abscess formation in at least one kidney, with most (87%) harboring bilateral abscess. In contrast, only 7% of females developed unilateral abscess ( $P<0.0001$  vs. female proportion). Males surgically infected with UPEC urosepsis isolate CFT073 likewise exhibited abscess and significantly higher kidney bacterial titers 2 wpi than females ( $P=0.0002$ ; **Figure 11**).

Histologic examination of male C3H/HeN kidneys demonstrated extensive abscess formation in subcapsular, cortical, and medullary locations (**Figure 12**). These abscesses featured a dense neutrophilic infiltrate with areas of necrosis (**Figure 10C**). The surrounding renal parenchyma was abnormal throughout, exhibiting interstitial infiltrates, edema and fibrosis,

mesangial sclerosis, and tubular atrophy with visible thyroidization suggesting active, progressive renal disease (**Figure 10D**; **Figure 12**).

*Testosterone mediates UTI severity in male C3H/HeN mice.*

The observed susceptibility of male mice to chronic cystitis, pyelonephritis and renal abscess could be attributable to a number of sex-specific factors. We adapted our model to examine the potential influences of sex steroid hormones. Females and males were gonadectomized or sham-operated at 5 weeks of age and allowed to recover completely over 4 weeks before mini-surgical UTI was introduced. We found no observable differences in bladder or kidney colonization 2 wpi between ovariectomized and sham-operated C3H/HeN females, suggesting that estrogens do not influence chronic cystitis risk (**Figure 13A**). However, bladder bacterial burdens in castrated males 2 wpi were significantly lower than in sham-operated controls ( $P=0.0049$ ; **Figure 13B**). The majority (87%) of sham-operated C3H/HeN males developed chronic cystitis, compared with only 27% of castrated males ( $P=0.0025$  vs. castrated proportion). Further, castrated males exhibited a bimodal distribution remarkably similar to C3H/HeN females. Castration similarly attenuated renal infection, reflected in significantly lower kidney bacterial titers in castrated animals compared to sham-operated controls ( $P<0.0001$ ; **Figure 13B**).

To specify whether male susceptibility to severe UTI is mediated by androgens or by another testicular factor, we implanted slow-release testosterone or placebo pellets 5 days after castration or sham operation. Consistent with above results, castration attenuated severe UTI in placebo-treated males (**Figure 13C**). However, treatment of castrated males with exogenous testosterone reversed the protective effects of gonadectomy; significantly higher bladder

( $P < 0.0001$ ) and kidney ( $P = 0.0002$ ) bacterial burdens were present in testosterone-treated castrated males compared to placebo-treated castrated males (**Figure 13C**). Testosterone-complemented castrated males were statistically indistinguishable from placebo-treated sham males. Collectively, these data indicate that androgens mediate male susceptibility to chronic cystitis and severe pyelonephritis.

## Discussion

In this study, we report the first preclinical UTI model to permit direct elucidation of sex differences in susceptibility and host response to infection, using a novel inoculation technique that bypasses anatomic differences in the lower urinary tracts of male and female mice. Acute cystitis in male mice proceeded through the IBC cascade in a fashion similar to females, but the incidence of chronic cystitis was strikingly higher in C3H/HeN males. Further, these males developed more advanced pyelonephritis and highly prevalent abscess formation compared to females. These phenotypes were mitigated in males who underwent gonadectomy prior to infection, but complemented with exogenous testosterone. Our findings indicate that sex influences on susceptibility to multiple forms of UTI are complex and not limited to traditionally cited anatomic differences. In addition, our mini-surgical model provides a new preclinical platform for translatable studies of upper- and lower-tract UTI pathogenesis in both male and female hosts.

To successfully colonize the urinary tract, UPEC must circumvent formidable host defenses, which are mechanical, biochemical, and immunologic in nature. In ascending UTI, UPEC evades these defenses, in part, by following a well-documented pathogenic cascade keyed by the formation of IBCs in the bladder (15-21, 23-26). Our data offer the first evidence of the IBC



pathway in males and indicate that the events comprising acute cystitis are fundamentally similar in both sexes. Males that resolved acute infection also maintained a small population of persisting UPEC within bladder tissue, likely representing the quiescent intracellular reservoirs believed to seed same-strain recurrent infection in females (42).

Male C3H/HeN mice also showed greater susceptibility to chronic cystitis than females, with chronically infected animals of both sexes displaying similar histopathology and inflammatory profiles. The chronic cystitis phenotype has proven to be an important outcome in female models investigating the natural history of UTI and host responses to repeated UPEC inoculation (29, 30, 41). Our data support previous research in catheter-infected female mice demonstrating that infection outcome is determined within 24 hpi via an acute host inflammatory checkpoint (29). Thus, in both female and male hosts, the nature and degree of early host inflammatory responses may specify risk for severe or recurrent UTI.

Sex differences in immune response have been demonstrated in a number of human infections and animal models; in many of these studies, females display more robust resistance to pathogens (47-49). Among other hypotheses, this enhanced defense has been attributed to an evolutionary need for pregnant females to protect their fetuses from infection (50) and has been linked to women's higher incidence of autoimmune and chronic inflammatory diseases (51, 52). Germane to the present study, UTI are the most common bacterial infections of women(9) and females of other animal species (53-55), and UPEC strains isolated from women or female animals share considerable phylogenetic similarity and parallel virulence profiles (54, 55). Heightened defenses in the female urinary tract may thus have evolved in response to continual exposure to bacterial pathogens (*e.g.*, following sexual intercourse (11)). Consistent with this paradigm, female mice more aptly controlled both lower and upper UTI compared to males in

our model. Additionally, females displayed higher local proinflammatory cytokines 24 hpi (when bladder UPEC burdens were similar between sexes), suggesting that a more pronounced acute inflammatory response may be raised in the female urinary tract to better control infection.

In addition to potential evolutionary pressure to repel urinary pathogens, some evidence has suggested that estrogen may impact immune response to UTI, although the influence of male androgens on UTI outcome has not been explored. The incidence of UTI in women increases following menopause (56), and studies in ovariectomized C57BL/6 mice suggest that diminished estrogen may favor bacterial persistence in the bladder (57, 58). Here, we found that ovariectomy did not affect chronic infection outcome in C3H/HeN females, but androgens potentiated the development of chronic cystitis and severe pyelonephritis in males. The exact organizational or activational influences of androgens on UTI severity in both males and females represent areas of ongoing investigation. Notably, our findings suggest that clinical modulation of androgens may represent a potential therapeutic route to combat recalcitrant or recurrent UTI.

At a glance, our findings in this new model may appear to represent a stark contradiction to the female predominance in human UTI epidemiology. However, mini-surgical inoculation bypasses anatomic sex differences below the bladder. Thus, our data are in fact consistent with the long-standing presumption that UTI risk in women is potentiated by anatomic features. Conversely, anatomy represents a key defense against UTI in males, and the evolutionarily “naïve” urinary tract of males may provide a more hospitable environment for UPEC and other uropathogens once these male anatomic barriers are bypassed. This paradigm is consonant with clinical data reflecting increased morbidity and mortality in men who develop pyelonephritis and complicated UTI, compared to women with these conditions (2-5). While acute cystitis was equivalent in males and female mice, chronic cystitis progression was much higher in males and

associated with marked renal infection, leading us to speculate that severe UTI in the male mouse is driven primarily by testosterone-induced susceptibility within the kidney.

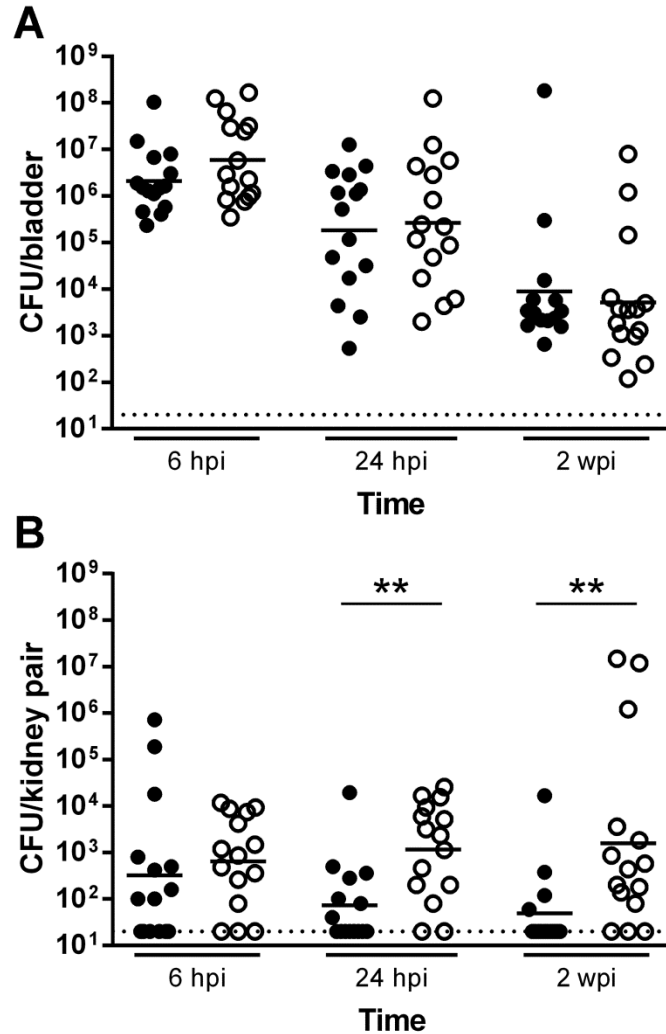
In addition to facilitating the study of sex differences, our mini-surgical model fills another substantial gap in the basic investigation of UTI. No published approaches in experimental UTI result in more than a very small minority of wild-type animals developing ascending renal abscess (29, 59, 60). Consequently, the field has lacked ability to model ascending abscess development and the resolution of severe renal infection with treatment. In the present model, most C3H/HeN males develop severe pyelonephritis and exhibit renal abscesses, judged to arise via the ascending route (rather than hematogenous, as we detected no bacteria in blood cultures). As noted above, C3H mice feature enhanced vesicoureteral reflux compared to C57BL/6 mice (43, 46), reflecting a risk factor for pyelonephritis that is also commonly present in the human population. Our work therefore opens a new avenue into modeling the development, therapy, resolution, and sequelae of severe pyelonephritis and ascending renal abscess. Beyond these niches, our method also induces visible infection of the prostate, making the model potentially useful for the study of bacterial prostatitis.

In summary, the work described here will accelerate fundamental investigation into the mechanisms of UTI initiation, progression, and persistence in male hosts, as well as expanding studies of pyelonephritis, ascending renal abscess, and the treatment of complicated UTI. These advances also represent a timely response to recent calls by the National Institutes of Health (61) and in the basic and clinical literature (62-64) for sex-based research approaches to infectious and other diseases, including a specific focus on UTI (65, 66). Continued work in this model promises to address these initiatives at a mechanistic level and to generate translatable advances in prevention and treatment of these common infections.

## **Acknowledgements**

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## Figures



**Figure 1. Direct bladder inoculation reliably infects the urinary tract of C57BL/6 mice.**

Female (●) or male (○) C57BL/6 mice were surgically infected with  $10^7$  CFU UTI89. (A)

Bladders were homogenized at the indicated time points, serially diluted, and CFU enumerated.

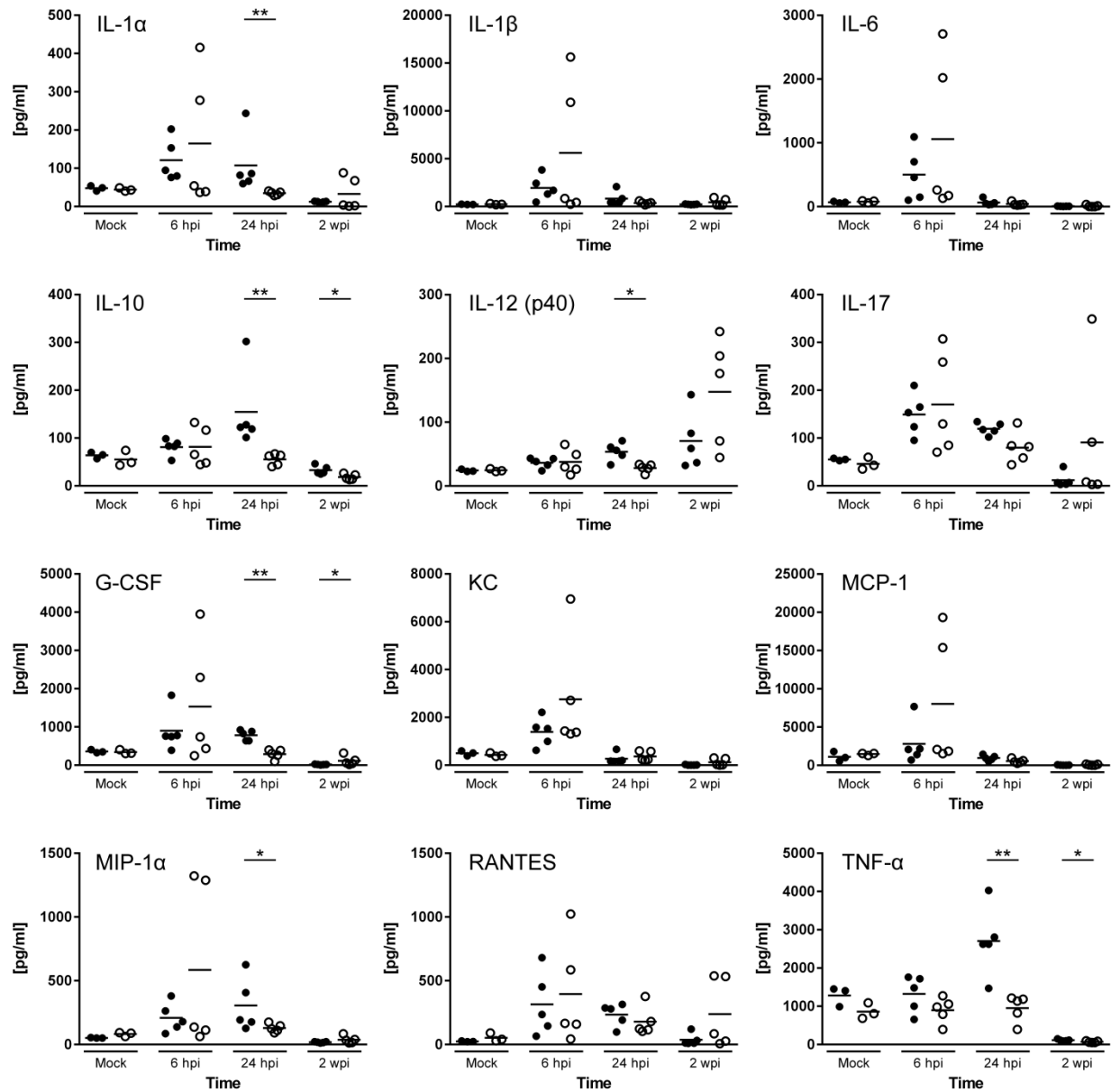
Each point represents one mouse; bars indicate geometric mean. Data comprise three

independent experiments of 5 mice per group. Dotted line represents the limit of detection. (B)

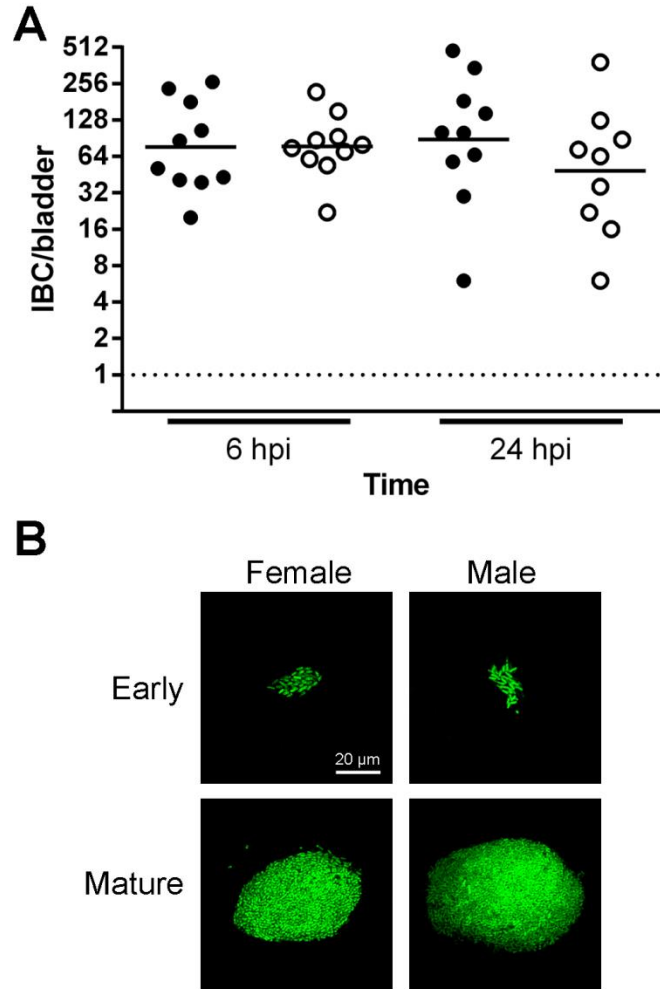
Total bacteria present in both kidneys were enumerated. Aggregate data from three independent

experiments with 5 mice per group are shown. Statistically significant differences between sexes

are indicated (\*\*  $P < 0.01$ ).



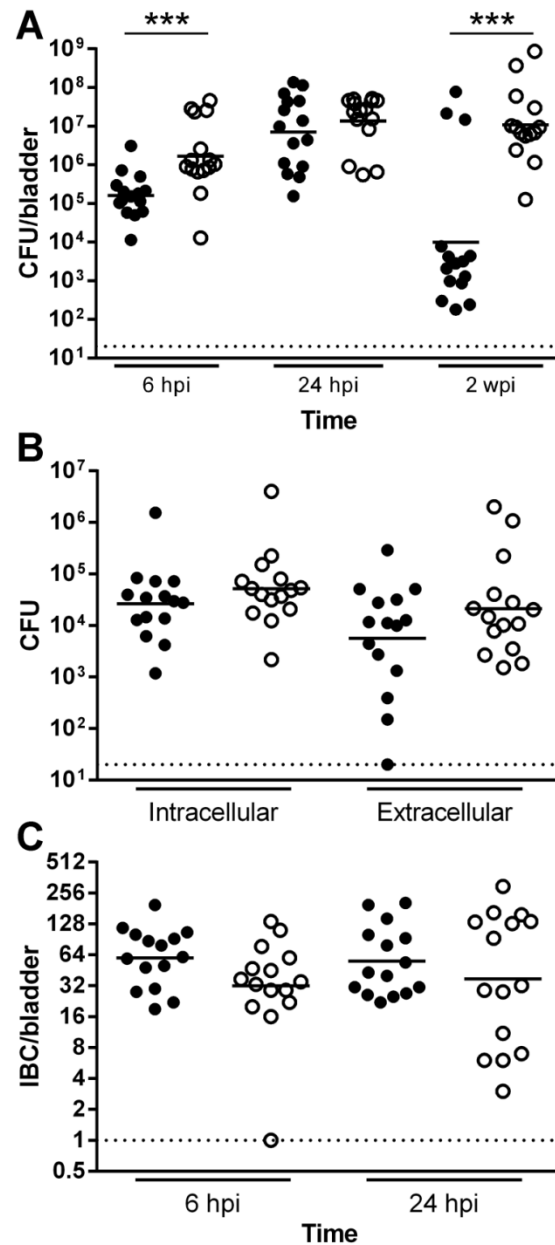
were assayed in duplicate. Statistically significant differences between sexes are indicated (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ).



**Figure 3. Cystitis in male C57BL/6 mice follows the IBC pathway.** Female (●) or male (○) C57BL/6 mice were surgically infected with  $10^7$  CFU UTI89. **(A)** IBCs present in bisected bladders 6 or 24 hpi were stained with X-gal and enumerated. Data from two independent experiments with 5 mice per group are shown; bars indicate geometric mean. Dotted line represents the limit of detection. There were no statistically significant differences between sexes at either time point. **(B)** The morphology of IBCs in female (left) and male (right) C57BL/6 were observed 16 hpi with GFP-expressing UTI89 via confocal microscopy. In both sexes, early IBCs appeared as small (20 μm diameter) collections of intracellular bacteria with

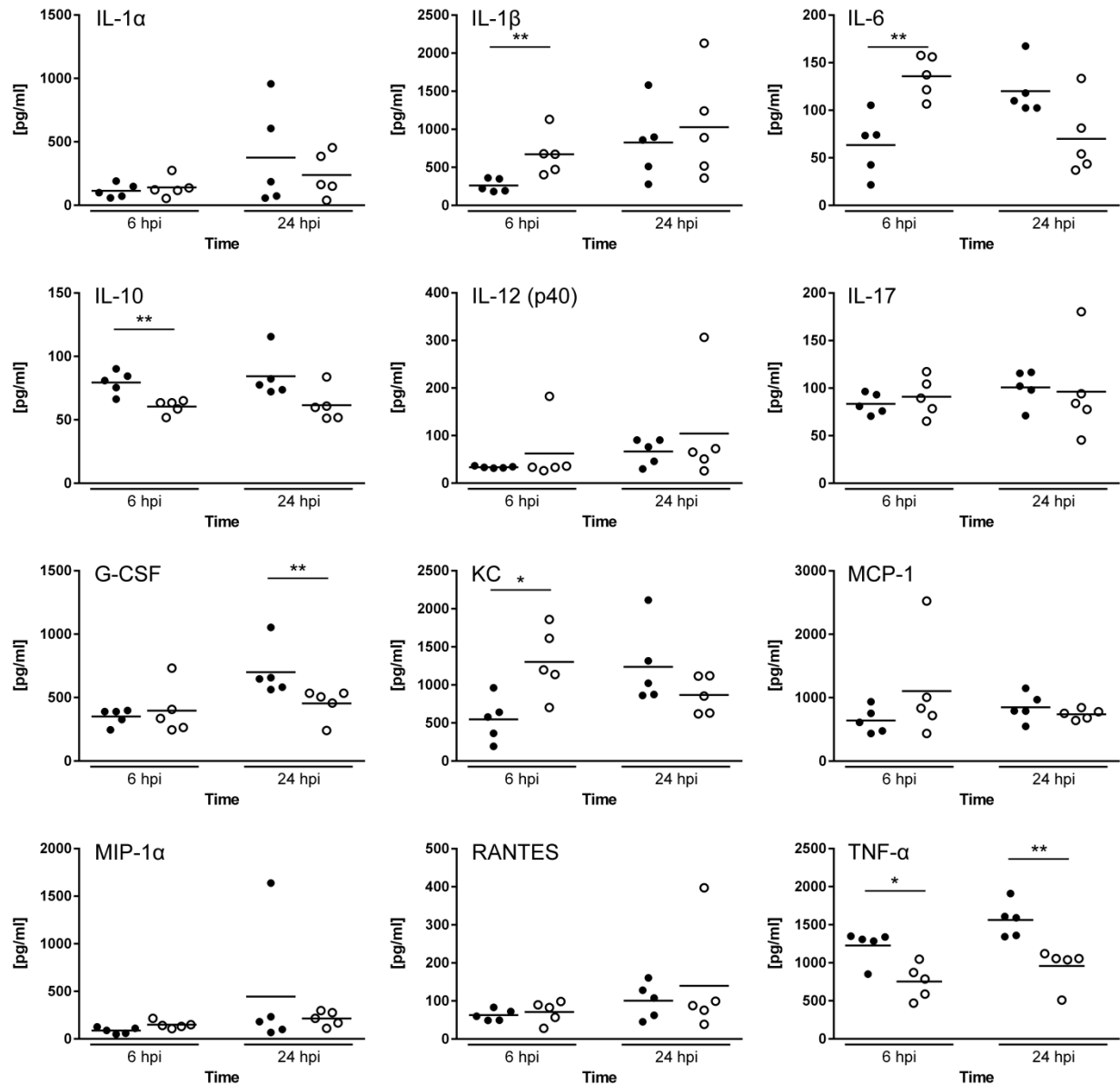


bacillary morphology, while mature IBCs comprised coccoid UPEC in >100- $\mu$ m masses  
Representative images of early (top) and mature (bottom) IBCs are shown.

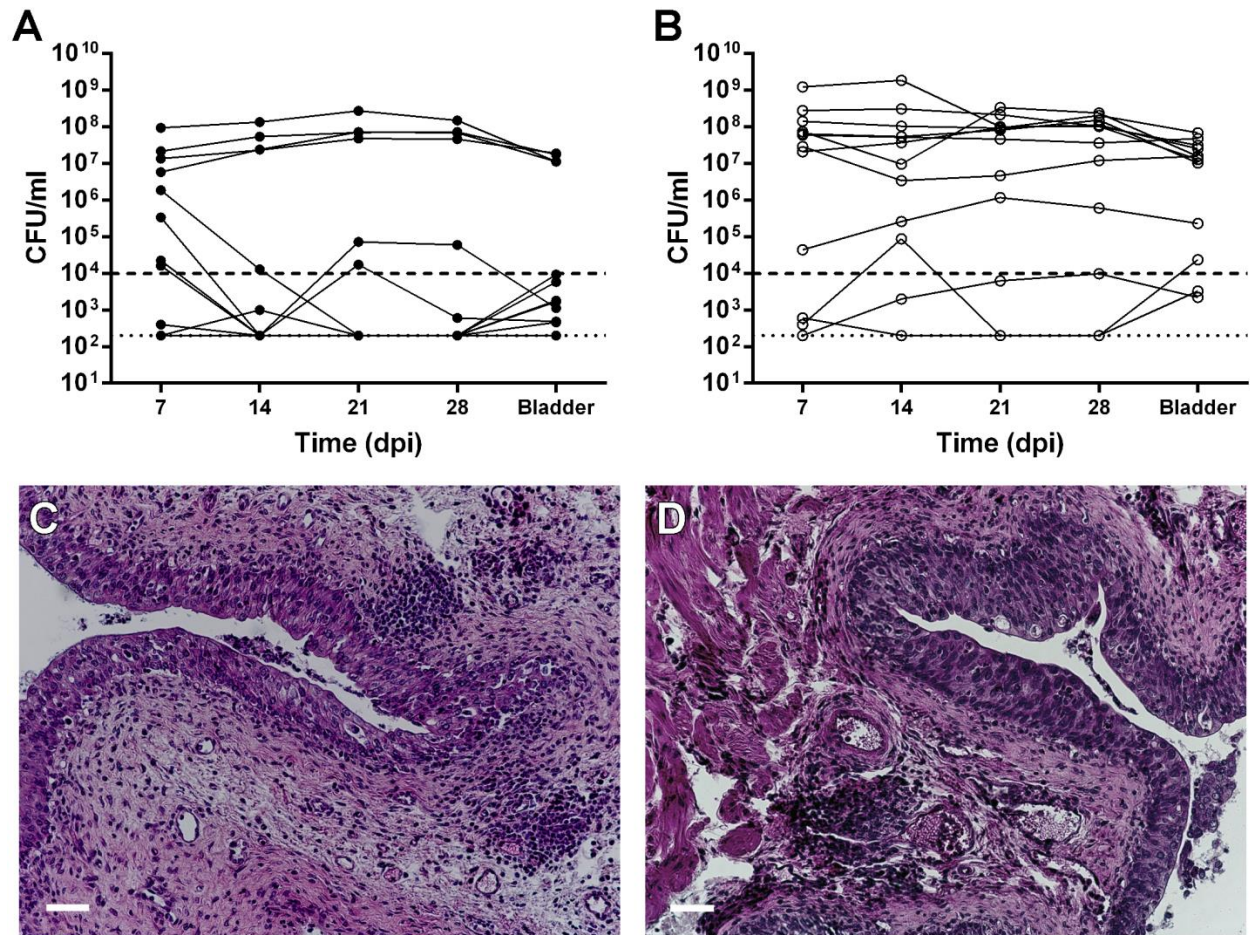


**Figure 4. UPEC more readily colonize the male C3H/HeN bladder.** Female (●) or male (○) C3H/HeN mice were surgically infected with  $10^7$  CFU of UTI89. (A) Bladders were homogenized at the indicated time points, serially diluted, and CFU enumerated. Each point represents one mouse; bars indicate geometric mean. Data comprise three independent experiments with 5 mice per group. Dotted line represents the limit of detection. Mice were classified as chronically infected if 2-wpi bladder titers were  $> 10^4$  CFU/bladder. Statistically

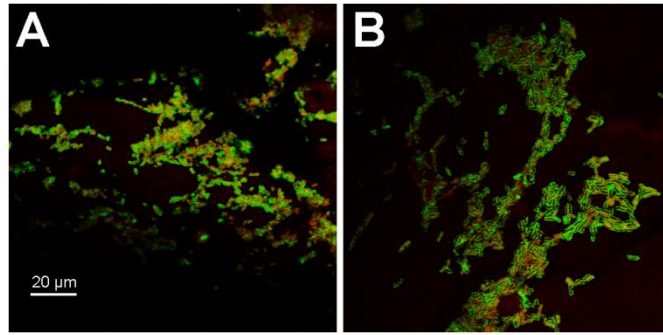
significant differences between males and females are indicated (\*\*\*)  $P < 0.001$ ). **(B)** Bacteria present in the intracellular (gentamicin-protected;  $P=0.18$ ) or extracellular (gentamicin-sensitive;  $P=0.38$ ) compartments of bladders from female (●) or male (○) C3H/HeN mice at 6 hpi were enumerated. Aggregate data from three independent experiments with 5 mice per group are shown. **(C)** IBCs present in bisected bladders 6 and 24 hpi in female (●) or male (○) C3H/HeN mice were enumerated by microscopy following X-gal staining. Data from three independent experiments with 5 mice per time point are shown.



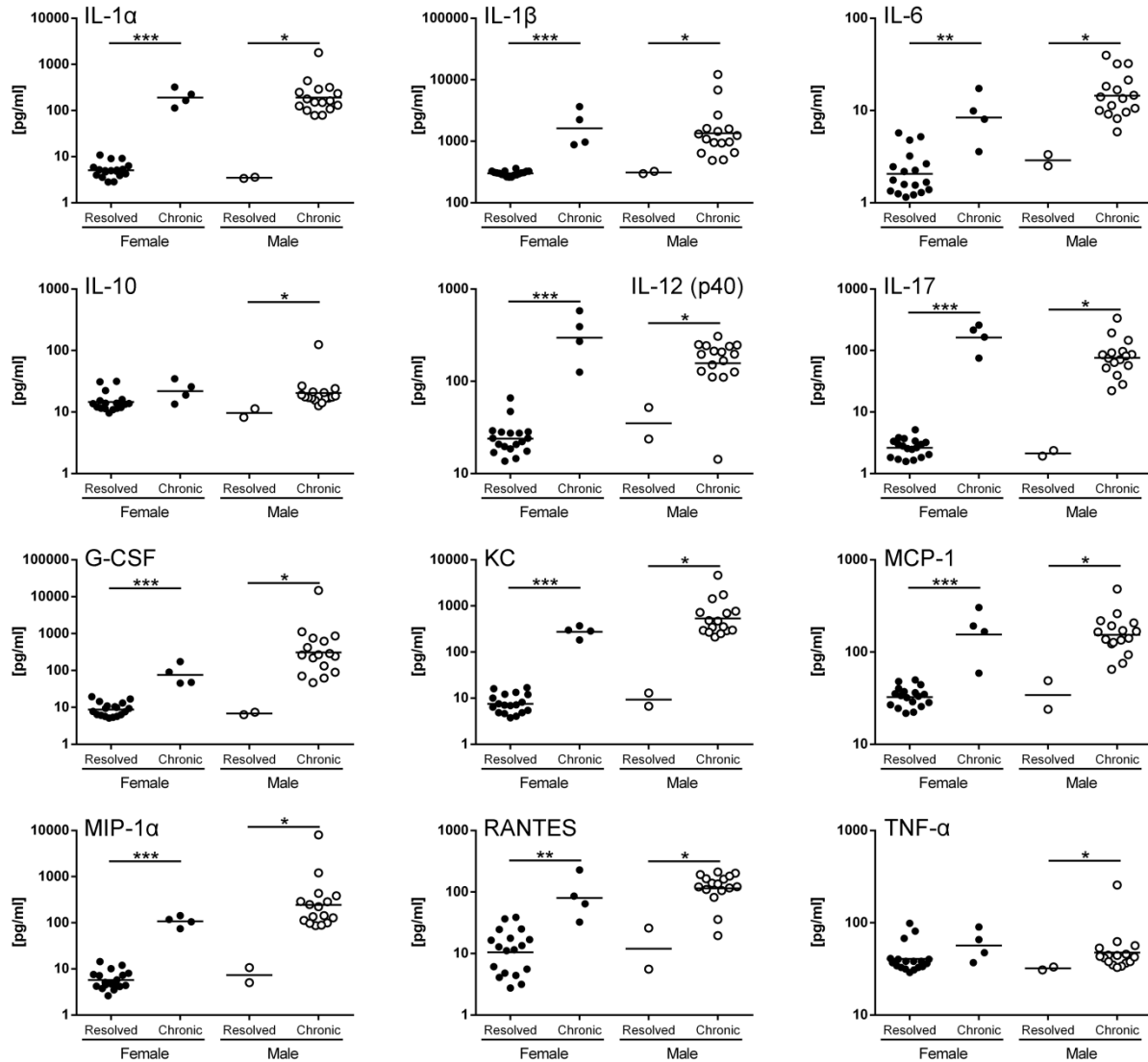
**Figure 5. Male and female C3H/HeN hosts exhibit modest differences in bladder cytokine expression during acute cystitis.** Female (●) or male (○) C3H/HeN mice were surgically infected with UTI89. Bladders were homogenized 6 or 24 hpi and centrifuged to remove cellular debris before cytokine expression was measured. Bars represent the mean from 5 individual mice per time point from two independent experiments. Samples were assayed in duplicate. Statistically significant differences between sexes are indicated (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ).



**Figure 6. Male C3H/HeN mice develop chronic cystitis.** Female (●) and male (○) C3H/HeN mice were surgically infected with  $10^7$  CFU of UTI89 and monitored for 4 wpi. (A and B) Weekly urine bacterial titers and corresponding 4 wpi bladder titers were enumerated in female (A) and male (B) mice following infection. Solid lines connect corresponding urine titer time points and 4 wpi bladder titers (Bladder) from each individual mouse. Dashed horizontal lines represent the cutoff for significant bacteriuria ( $10^4$  CFU/ml). Dotted horizontal lines show the limit of detection. Data were compiled from 2 independent experiments. (C and D) Paraffin-embedded bladder sections 4 wpi from persistently bacteriuric female (C) and male (D) C3H/HeN mice were examined by hematoxylin & eosin and light microscopy. Scale bars approximate 50  $\mu$ m.



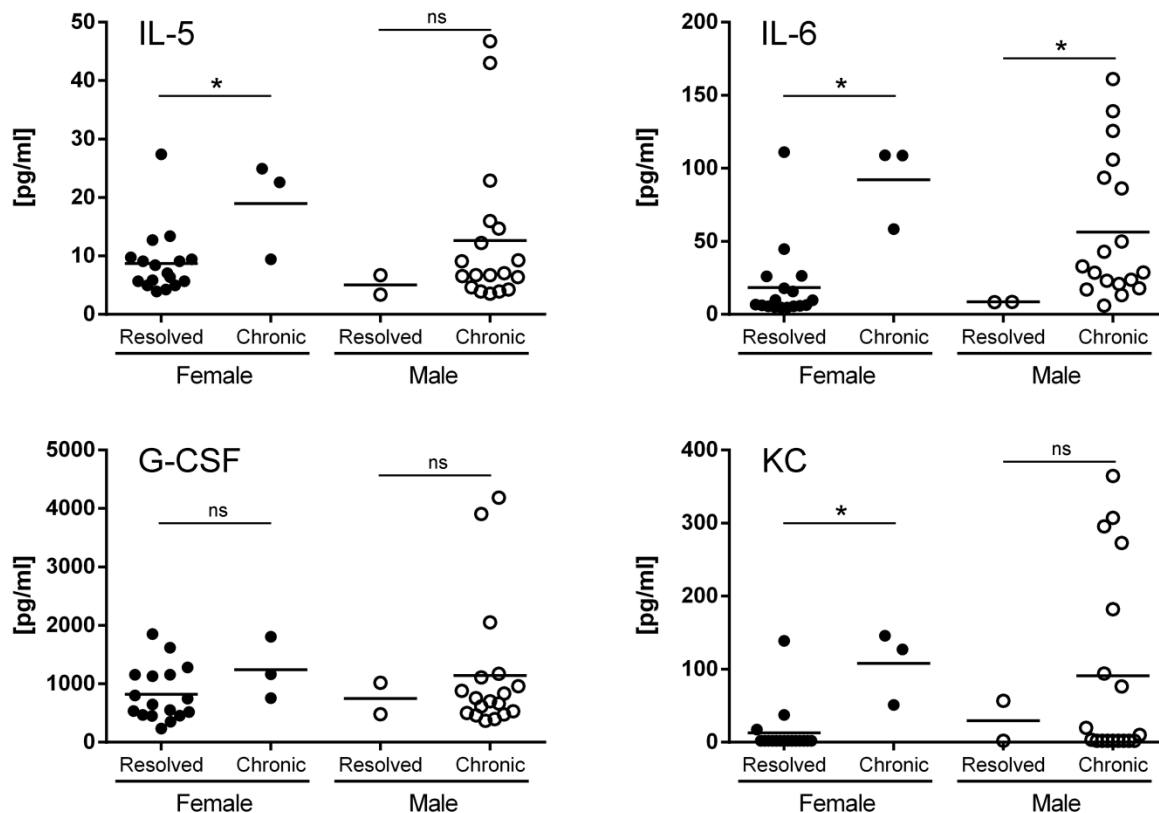
**Figure 7. UPEC display similar bacterial morphology and luminal colonization in chronically infected male and female C3H/HeN mice.** Female (A) or male (B) C3H/HeN mice were surgically infected with UTI89 and followed for 2 wpi. The bladders of mice that displayed persistent bacteriuria were examined via immunofluorescence and confocal microscopy; representative images are shown.



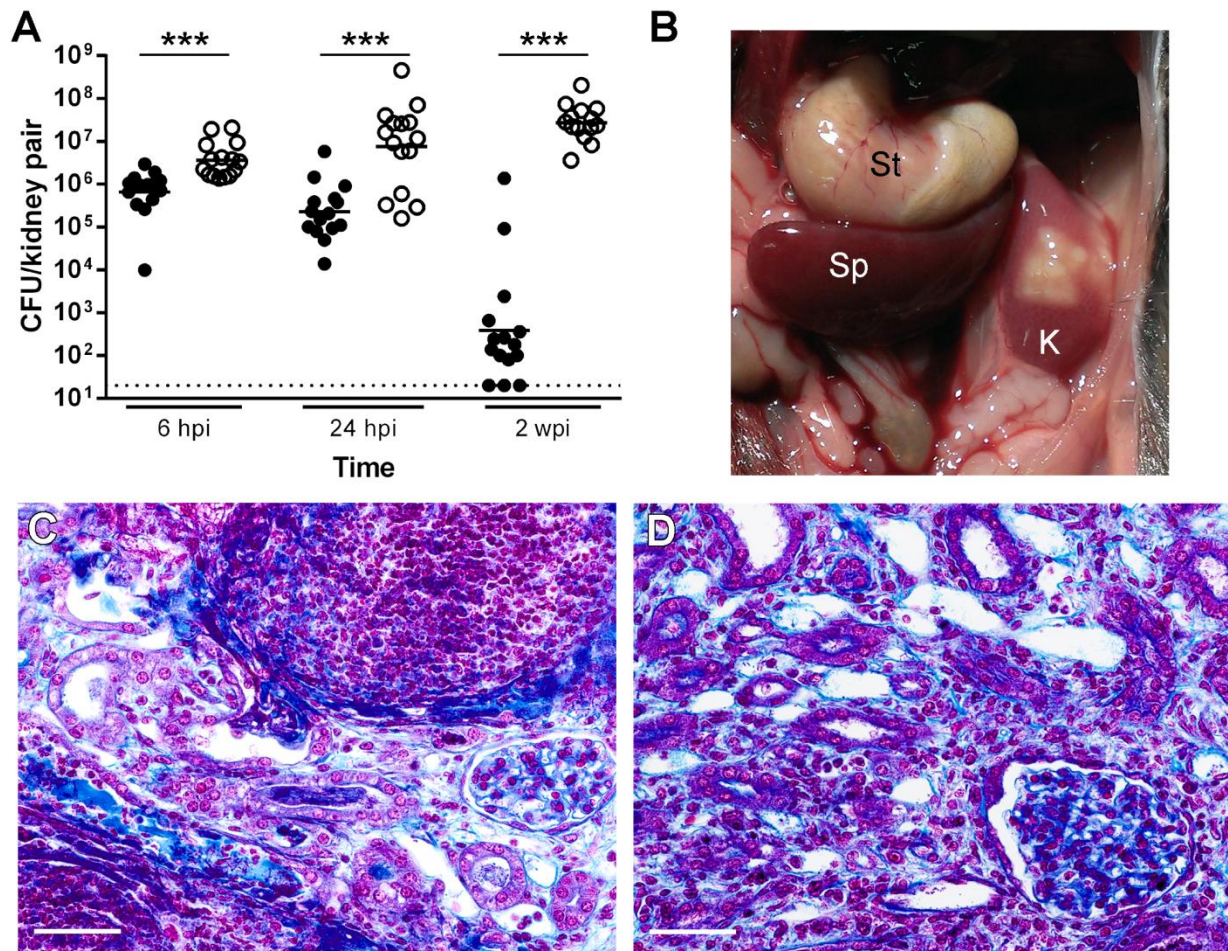
**Figure 8. Female and male mice with chronic cystitis display similar elevations in bladder pro-inflammatory cytokines.** Female (●) and male (○) C3H/HeN mice were surgically infected with  $10^7$  CFU UTI89 and followed for 2 wpi. Bladders were homogenized and centrifuged to remove cellular debris before cytokine expression was measured. Mice were grouped as resolved or chronic, as outlined in Methods. Bars represent geometric means, with each point indicating an individual mouse, assayed in duplicate. Data are shown from three independent experiments. Statistically significant differences between chronic and resolved groups are

indicated (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ). No significant differences were observed between sexes.



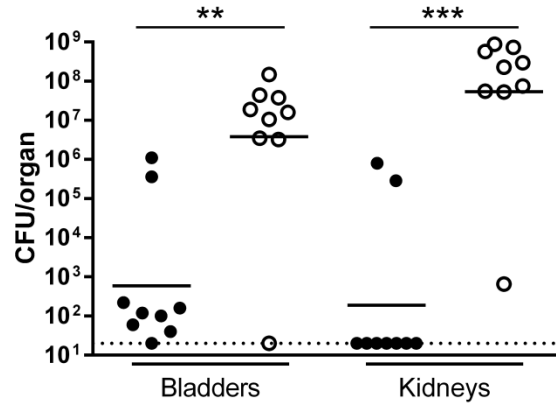


**Figure 9. Male C3H/HeN mice display acute inflammatory biomarkers predictive of chronic outcome.** Female (●) and male (○) C3H/HeN mice were surgically infected with  $10^7$  CFU of UTI89 and monitored for 2 wpi. Sera was collected 24 hpi and cytokine content measured by multiplex bead array. Mice were grouped as resolved or chronic, as outlined in Methods. Bars represent geometric means, with each point indicating an individual mouse, assayed in duplicate. Aggregate data from three independent experiments are shown. Statistically significant differences between chronic and resolved groups are indicated (\*  $P < 0.05$ ). No significant differences were found between sexes. Statistical comparisons in male serum IL-5, G-CSF, and KC were likely limited by the lack of  $n$  of resolved males.

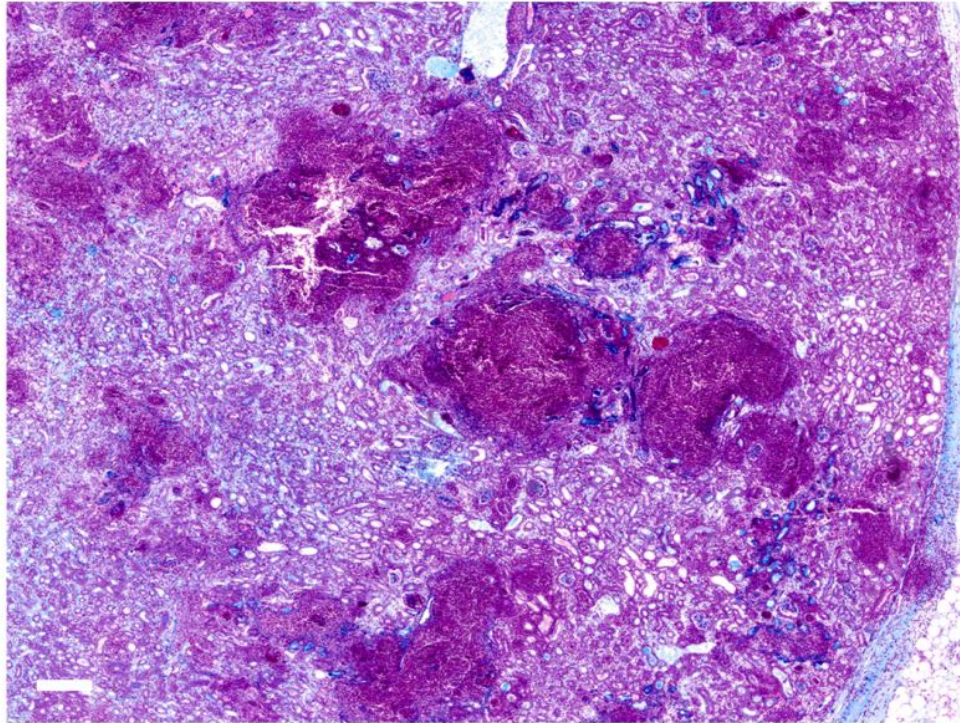


**Figure 10. Male C3H/HeN mice develop severe pyelonephritis and 100% penetrant renal abscess.** Female (●) and male (○) C3H/HeN mice were surgically infected with  $10^7$  CFU UTI89. (A) Kidney pairs were homogenized, serially diluted, and CFU enumerated at the indicated time points. Each point represents one mouse; bars indicate geometric mean. The dotted horizontal line represents the limit of detection. Data comprise three independent experiments of 5 mice per group. Statistically significant differences between sexes are indicated (\*\*\*  $P < 0.001$ ). (B) Following autopsy, gross renal abscesses were observed in male, but not female, C3H/HeN mice 2 wpi. A representative image of the stomach (St), spleen (Sp) and abscessed left kidney (K) of a male C3H/HeN mouse 2 wpi is shown. (C and D) Paraffin-embedded kidney sections 2 wpi from male C3H/HeN mice were examined by Gomori

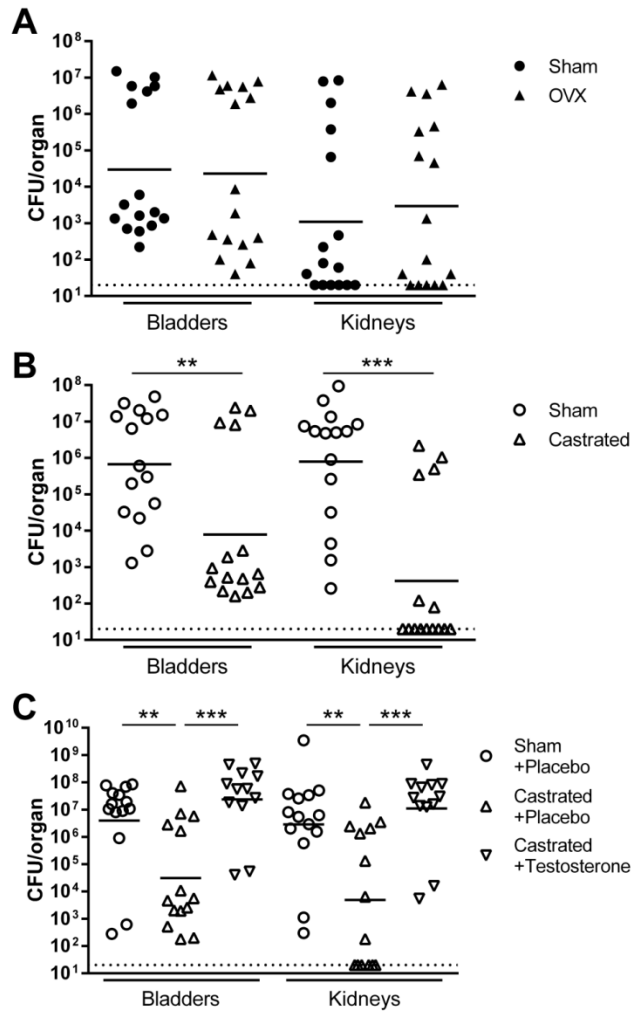
trichrome stain and light microscopy. Representative images illustrate (C) areas of necrotic abscess and (D) diseased renal cortex; areas of collagen deposition appear blue (scale bars, 50  $\mu\text{m}$ ).



**Figure 11. Male C3H/HeN surgically infected with a urosepsis isolate, CFT073, develop more severe chronic UTI.** Female (●) or male (○) C3H/HeN mice were surgically infected with 10<sup>7</sup> CFU of CFT073. Bladders (left) and kidneys (right) were homogenized 2 wpi, serially diluted, and CFU enumerated. Eight of 9 males had visible renal abscess formation. Each point represents one mouse; bars indicate geometric mean. Data comprise two independent experiments with 4 or 5 mice per group. Dotted line represents the limit of detection. Statistically significant differences between males and females are indicated (\*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).



**Figure 12. Male C3H/HeN develop severe renal abscess and fibrosis.** Male C3H/HeN mice were surgically infected with  $10^7$  CFU UTI89. Paraffin-embedded kidney sections 2 wpi were examined by Gomori trichrome stain and light microscopy. Representative image at low-power magnification illustrates cortical and medullary abscesses. Areas of collagen deposition appear blue. Scale bar, 200  $\mu$ m.



**Figure 13. Testosterone enhances susceptibility to chronic UTI.** (A) Sham-operated (●) and ovariectomized (OVX; ▲) C3H/HeN female mice were surgically infected with  $10^7$  CFU of UTI89 4 weeks post-operatively (at 9 weeks of age). Bladders (left) and kidney pairs (right) were homogenized, serially diluted, and CFU enumerated 2 wpi. Each point represents one mouse; bars indicate geometric mean. Data comprise three independent experiments of 5 mice per group. (B) Sham-operated (○) and castrated (Δ) C3H/HeN male mice were surgically infected with  $10^7$  CFU of UTI89 four weeks post-operatively (9 weeks of age). Bladders (left) and kidney pairs (right) were homogenized, serially diluted, and CFU enumerated 2 wpi. Each point represents one mouse; bars indicate geometric mean. Data comprise three independent

experiments of 5 mice per group. The dotted horizontal line shows the limit of detection. (C) Sham-operated male C3H/HeN mice were subcutaneously implanted with slow-release placebo pellets 5 days post-operatively, and castrated males were implanted with placebo or testosterone pellets. Mice were surgically infected 4 weeks post-gonadectomy (at 9 weeks of age). Bladders (left) and kidney pairs (right) were homogenized, serially diluted, and CFU enumerated 2 wpi. Each point represents one mouse; bars indicate geometric mean. Data comprise three independent experiments of 4-5 mice per group. The limit of detection is indicated with the dotted horizontal line. Statistically significant differences between experimental groups are indicated (\*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).



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CHAPTER THREE

ANDROGEN RECEPTOR ACTIVATION ENABLES ASCENDING *ESCHERICHIA COLI*  
RENAL ABSCESS FORMATION

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**Abstract**

Females across the lifespan and certain male populations are susceptible to urinary tract infections (UTI). The influence of sex on UTI is incompletely understood, in part because preclinical modeling has been performed almost exclusively in female mice. In recent studies using a new mini-surgical bladder inoculation model, male C3H/HeN mice displayed enhanced



susceptibility to severe pyelonephritis; most males developed renal abscess, a complication rare in female mice. Here, we reveal that within developing renal abscesses, uropathogenic *Escherichia coli* (UPEC) formed dense intratubular, biofilm-like communities sheltered from infiltrating leukocytes by a fibrotic tubular epithelium. Formation of intratubular communities required type 1 pili, heretofore widely viewed as a virulence factor only in the bladder.

Administration of exogenous testosterone in C3H/HeN females also imparted susceptibility to severe pyelonephritis and abscess formation. Susceptibility in both sexes was potentiated with 5 $\alpha$ -dihydrotestosterone, and males with androgen receptor deficiency were protected from severe pyelonephritis, demonstrating that specific activation of the androgen receptor augments UTI severity in both sexes. Visualization of intratubular UPEC communities illuminates early renal abscess pathogenesis and portends new studies of host-pathogen interactions in this niche. Our data also suggest that androgen modulation may represent a novel therapeutic route to combat recalcitrant or recurrent UTI.

## **Introduction**

Bacterial infection of the urinary tract represents one of the most common human infectious diseases and imposes a taxing burden on society; approximately 10 million primary care provider visits annually are for uncomplicated lower urinary tract infection (UTI), incurring >\$2 billion in economic costs (1, 2). Ascension of uropathogens to the kidneys, resulting in pyelonephritis or urosepsis, is associated with mortality and threatens lifelong morbidity, including renal scarring and attendant risks of hypertension and chronic kidney disease, despite appropriate initial antibiotic treatment (3, 4). These contemporary challenges to the human host manifest in an era in which the primary causative agent of UTI, uropathogenic *Escherichia coli* (UPEC), displays

unprecedented global prevalence and breadth of antimicrobial resistance (5). It is therefore critical to understand bacterial and host mechanisms of disease in the kidney to illuminate novel therapeutic routes to combat these serious infections and their sequelae.

Preclinical modeling of ascending pyelonephritis has been constrained by a lack of susceptible female murine models and technical inability to access the male mouse bladder via catheter (6, 7). To decipher sex differences in the disease, we recently developed a minimally invasive surgical bladder inoculation model enabling direct comparison of male and female hosts. After equal intravesical inoculation with UPEC, male mice of multiple genetic backgrounds developed much more severe renal infection than females. While female mice rarely form kidney abscess, we observed macroscopic, bilateral renal abscess and extremely high kidney UPEC burdens in >90% of infected C3H/HeN males by 2 wk post infection (wpi) (7). The C3H background is known to exhibit vesicoureteral reflux (VUR), a translationally relevant feature because VUR is a major risk factor for upper-tract UTI, particularly in affected children (8). Moreover, micro- and macroscopic abscess formation is a pathological feature of pyelonephritis in humans (9), and the severity of inflammation correlates with subsequent renal scarring in several models of renal injury (10, 11). The present model therefore enables the discovery of niches and mechanisms exploited by UPEC for growth and persistence in the kidney en route to formation of renal abscess in the setting of ascending pyelonephritis.

We previously reported that castration in male C3H/HeN mice prior to induction of UTI mitigated the development of chronic cystitis, severe pyelonephritis, and renal abscess, while ovariectomy had no effect on chronic outcome in females (7). Furthermore, exogenous testosterone replacement in castrated male mice complemented the severe UTI phenotype, implicating androgens in susceptibility to severe UTI (7). As these findings were developed in

male mice, it was unknown if androgen-driven UTI susceptibility extends to females, in whom UTI is substantially more prevalent overall (1, 12, 13). Here, we interrogated the temporal course of bacterial localization within developing renal abscesses and defined hormonal signaling pathways that influence UTI susceptibility. UPEC formed dense, biofilm-like communities filling renal tubules at the center of developing abscesses, and were sheltered from surrounding inflammatory cells through fibrosis associated with tubular epithelia. These intratubular communities and associated severe pyelonephritis were induced in female mice by elevating androgen levels, phenocopying the course observed in male C3H/HeN mice. Further, pharmacological and genetic manipulation demonstrated that specific activation of the androgen receptor promotes development of severe pyelonephritis in both female and male hosts.

## **Results**

*Ascending renal abscesses emerge in male C3H/HeN mice in a narrow temporal window.*

As traditional preclinical modeling of UTI is limited to female mice, and females of most laboratory strains are highly resistant to pyelonephritis and renal abscess formation (6, 7, 14, 15), there is a significant gap in our understanding of the pathogenesis and development of ascending renal abscesses. However, by 2 wpi, over 90% of C3H/HeN males undergoing mini-surgical bladder inoculation develop gross bilateral renal abscess (7). This model can thus be used to interrogate the anatomic and temporal details of abscess onset in the kidney following intravesical inoculation of UPEC. We previously showed that surgically infected male C3H/HeN mice develop higher bladder and kidney UPEC burdens than females 24 hours post infection (hpi), but do not yet display renal abscesses at this interval (7). Here, we surgically infected male C3H/HeN mice with the cystitis-derived UPEC isolate UTI89 and monitored

abscess progression over time. Histological examination of kidneys 3 days post infection (dpi) from mice exhibiting urine and bladder bacterial titers  $>10^4$  CFU (predictive of the development of pyelonephritis (7)) demonstrated no evidence of abscess and only scant neutrophilic inflammation near the renal pelvis, with no apparent inflammation or tissue damage in the renal cortex or medulla (**Figure 1A** and **Figure 2**). Visualized bacteria were limited to the renal pelvis and calyces (**Figure 1B**, black arrowhead), and immunofluorescence microscopy identified no UPEC outside the renal pelvis (data not shown).

By 5 dpi, a small proportion of infected mice (4 of 15, 27%) exhibited grossly evident abscess at necropsy. Immunofluorescence microscopy at this time point revealed small intraluminal collections of bacillary UPEC, distributed from the collecting system (**Figure 1C**, white arrowheads) to the proximal tubule (**Figure 1D**, white arrowhead and **Figure 3**). The tubules housing many of these UPEC communities reflected cellular injury and were surrounded by small numbers of infiltrating interstitial neutrophils, though inflammation was not evident within the tubular lumina (**Figure 1E-G**). Nine of 10 (90%) infected males developed gross renal abscess by 6 dpi (**Figure 1H**), equivalent to the frequency observed at 2 wpi (7). Thus, ascending renal abscesses in this model develop precipitously between 5 and 6 dpi, signifying rapid UPEC colonization of the renal parenchyma in this time window.

*UPEC assemble an intratubular biofilm-like nidus within ascending renal abscesses.*

The microscopic organization of UPEC during renal abscess formation has not been elucidated. Complete sectioning and microscopic examination of kidneys from infected male C3H/HeN mice displaying persistent bacteriuria 2 wpi revealed populations of bacteria in the centers of dense neutrophilic lesions (**Figure 4A**). Abscesses appeared predominantly at the

corticomedullary junction, with the PMN lesions surrounded by a rim of necrotic material (**Figure 4A**). UPEC were located entirely within the intratubular space, forming biofilm-like communities and adopting a tightly packed coccoid morphology by 2 wpi (**Figure 4B, C**) in contrast to their loosely associated and bacillary morphology 5 dpi (**Figure 1G**). Staining with anti-*E. coli* antibodies confirmed that UPEC comprised the entirety of this intratubular nidus at the center of renal abscesses (**Figure 4C**) and were rarely identified outside of the tubular lumen. Frequently, we observed multiple, discrete intratubular bacterial collections in close proximity within the same neutrophilic lesion (**Figure 4D**), suggesting that UPEC colonized some length of interconnected nephrons. Viewed at low power, kidney segments involved with inflammation distinctly followed a wedge-shaped pattern, while other areas of kidney were spared (**Figure 4E**). In conjunction with findings at earlier time points (**Figure 1**), these data suggest that ascending UPEC gain access to and colonize an isolated papillary collecting duct pyramid and (subsequently) multiple associated nephrons via robust intraluminal replication to nucleate abscess formation within a given segment of the kidney.

Abscesses rarely develop in female C3H/HeN mice (6, 7, 16), but we did identify an abscess with identical bacterial intratubular community morphology in one female mouse (out of >30 examined 2 wpi; **Figure 5**). Thus, bacterial organization within renal abscesses was preserved between sexes, but male sex conferred a dramatically increased risk for this upper-tract complication following UPEC inoculation of the bladder.

#### *Tubular fibrosis separates intratubular UPEC communities from recruited phagocytes.*

By 2 wpi, recruited neutrophils filled the renal interstitium surrounding UPEC-colonized tubules (**Figure 4**). Nearby tubules were largely destroyed or replaced by leukocytic infiltrates

or demonstrated other histologic signs of injury (*e.g.*, thyroidization and atrophy), while tubules harboring bacterial communities retained their structure (**Figure 4B, D**). Gomori trichrome staining indicated that infected tubules were fibrotic and encapsulated the UPEC abscess communities (**Figure 6A**, arrowheads). Most of these colonized tubules displayed no PMNs within the tubular lumen or bacterial mass (**Figure 4 and 6A**); when intratubular phagocytes were occasionally identified, they were associated with a breach in the fibrotic epithelial capsule (**Figure 6B, C**). Thus, we posit that UPEC infection provokes fibrosis of the tubular epithelium, in turn protecting the bacterial nidus from phagocytic attack.

Notably, UTI89-infected male C3H/HeN mice did not succumb to their severe infection, surviving for at least 30 wpi despite persistence of high renal bacterial burdens and abscesses, and maintaining sterile blood and spleen cultures (data not shown). Thus, while male kidney infection with UTI89 in this model is not self-limiting, it also does not result in lethal sepsis. It has been previously reported that mice with functional deficiency of Toll-like receptor 4 (TLR4), which exhibit a markedly delayed neutrophil response to UTI, do not develop renal abscesses even following direct renal injection of UPEC (6, 17, 18). We tested how TLR4 deficiency would impact ascending renal infection by surgically infecting male and female mice of the C3H/HeJ strain, insensitive to lipopolysaccharide owing to a TLR4 mutation. At 3 dpi, infected male C3H/HeJ mice displayed significantly higher renal ( $P=0.0010$ ) and bladder ( $P=0.0010$ ) bacterial burdens compared to female C3H/HeJ mice (**Figure 6D**) and did not exhibit visible renal abscess. Infected males succumbed to infection between 3 and 7 dpi, while most females survived through the period of observation (2 wpi) ( $P=0.0274$ ; **Figure 6E**). Of note, the timing of demise in infected male C3H/HeJ mice coincided with the window of abscess development we observed in C3H/HeN mice (see **Figure 1**). No infected C3H/HeJ males displayed gross

renal abscess at post-death necropsy or (in the minority that survived) at 2 wpi, despite all having kidney bacterial burdens  $>10^7$  CFU. Thus, TLR4-mediated signaling, expression, or activation are not responsible for sex differences in UTI; more importantly, abscess formation in the TLR4-competent host protects from lethal systemic infection. Collectively, these data support a model in which innate cellular responses and tubular fibrosis enable the host to contain infection and prevent systemic dissemination; however, this strategy also generates a sheltered intratubular niche for UPEC following their ascension from the bladder.

*Type 1 pili are required for renal abscess formation.*

Arriving in the female bladder, UPEC exploit mannose moieties decorating the luminal epithelial surface as the receptor for their major virulence determinant, the adhesive type 1 pilus; absence of type 1 pili or the tip adhesin FimH abolishes bacterial attachment and abrogates cystitis (19, 20). However, a role for type 1 pili in pyelonephritis has not been extensively explored. We used our surgical bladder inoculation model to investigate if type 1 pili were required for formation of intratubular UPEC communities within the kidney. Male C3H/HeN mice surgically inoculated with UTI89 $\Delta$ *fimH* resolved infection by 2 wpi, with markedly lower bladder and kidney bacterial loads (many sterile) in UTI89 $\Delta$ *fimH*-infected mice ( $P<0.0001$  vs. WT UTI89; **Figure 7A**). While all males infected with WT UTI89 developed gross renal abscess, we observed no abscess development in the kidneys of UTI89 $\Delta$ *fimH*-infected mice ( $P<0.0001$  by Fisher exact test). These marked differences in infection outcome were evident despite similar numbers of UTI89 and UTI89 $\Delta$ *fimH* reaching the male kidneys by 1 h after bladder inoculation ( $P=0.975$ ; **Figure 8**), demonstrating that type 1 pili are required for colonization of the kidney and formation of intratubular communities in this model.

Immunofluorescence microscopy confirmed that type 1 pili are expressed by UPEC within intratubular abscess communities 2 wpi (**Figure 7B**).

*Androgen exposure aggravates UTI severity in female C3H/HeN mice.*

As detailed above, castrated C3H/HeN males (like C3H/HeN females) rarely developed renal abscess, but susceptibility to severe UTI was restored by testosterone complementation (7). However, it was unclear if elevated testosterone levels in females could likewise amplify UTI severity and enhance abscess development. To address this question, we implanted slow-release testosterone or placebo pellets in female C3H/HeN mice 4 wk prior to induction of UTI. As expected, testosterone pellet implantation significantly increased serum testosterone compared to placebo-treated females, in fact mirroring serum testosterone levels seen in WT males of similar age (21, 22) (**Figure 9A**). Treatment of females with exogenous testosterone induced severe UTI 2 wpi (**Figure 9B**); significantly higher bladder ( $P=0.0102$ ) and kidney ( $P=0.002$ ) bacterial burdens were present in testosterone-treated females compared to placebo-treated controls. Consistent with prior results, a minority (36%) of placebo-treated females developed chronic cystitis, compared to 64% of androgenized females. More strikingly, androgen treatment led to development of gross abscess in at least one kidney in 64% of females, compared to 0 of 14 placebo controls ( $P=0.0006$ ). Abscesses in androgenized females appeared grossly and microscopically identical to those found in C3H/HeN males (7), recapitulating the time course and appearance of intratubular UPEC communities. Of note, while our surgical inoculation model does induce acute and chronic prostatitis with UTI89 in C3H/HeN males ((7) and **Figure 10**), the prostate does not contribute anatomically or otherwise to androgen-induced severe UTI (as testosterone-treated females, of course, lack prostates). In total, these data illustrate that



elevated circulating testosterone predisposes to severe UTI and renal abscess formation independent of biological sex.

*Androgen receptor activation drives UTI severity.*

We next aimed to identify the signaling pathway(s) by which testosterone acts to induce severe UTI. It is plausible that testosterone supplementation would perturb levels of other hormones in the hypothalamic-pituitary-gonadal (HPG) axis via its negative feedback on gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). In this scenario, increased GnRH, LH, and/or FSH could be protective in castrated males, while decreased secretion of these hormones in androgenized hosts could promote UTI susceptibility. Refuting this possibility, we previously found that while orchiectomy in males dramatically attenuated UTI, ovariectomy in females (which would activate the HPG axis similarly to castration in males (23)) had no influence on UTI outcome (7). This finding in conjunction with our ability to induce severe UTI in females via androgen treatment (**Figure 9B**) eliminates alteration in GnRH, LH, or FSH levels as a cause of increased UTI severity in androgenized hosts.

There are two primary routes by which testosterone could drive regulatory changes which lead to enhanced UTI. In one pathway, testosterone could undergo peripheral aromatization by aromatase, yielding estradiol which would then activate the estrogen receptor. To specify if this was the case, we tested whether an aromatase-resistant and more potent androgen receptor (AR) agonist, 5 $\alpha$ -dihydrotestosterone (DHT), could complement UTI severity in castrated males. We implanted slow-release DHT or placebo pellets 1 day after castration or sham operation in C3H/HeN males, then induced UTI 4 wk later. Consistent with our previous results, castration

attenuated bladder ( $P=0.041$ ) and kidney ( $P=0.0063$ ) bacterial burdens 2 wpi in placebo-treated males (**Figure 11A**). Treatment of castrated males with exogenous DHT reversed the effects of castration (**Figure 11A**), mirroring the complementation seen with testosterone (7). Organ bacterial burdens in castrated, DHT-treated males were similar to those in sham-operated, placebo-treated males, with both groups developing bilateral gross renal abscess in the majority of animals. Likewise, treatment of female C3H/HeN mice with DHT led to significantly higher bladder bacterial burdens 2 wpi ( $P<0.0001$ ) and a higher frequency of chronic cystitis ( $P=0.0025$  by Fisher exact test) in comparison to placebo-treated females (**Figure 11B**). DHT treatment also induced dramatically increased kidney titers (**Figure 11B**,  $P<0.0001$ ), and abscesses formed in 73% of these females ( $P<0.0001$  vs placebo). Thus, peripheral aromatization of testosterone does not mediate susceptibility to severe UTI. Of additional note, mice in these female-only experiments were infected via the traditional catheter route, demonstrating that the observed severe UTI phenotypes and their androgen dependence are not attributable to any aspect of the surgical infection methodology.

While classical AR activation is the most robust mechanism of androgenic signaling, new alternative signaling pathways that alter transcription independent of the androgen receptor have recently emerged (24, 25). To test classical AR involvement, we first undertook treatment with flutamide, an imperfect AR antagonist (26, 27). Flutamide administration over 4 wk prior to infection offered modest protection to C3H/HeN males 2 wpi compared to placebo-treated males in the bladder ( $P=0.0027$ ) and kidney ( $P=0.0047$ ) (**Figure 12A**), but did not attenuate disease to the degree seen following castration (7). We next adopted a genetic approach, using testicular feminization (Tfm) mice, which encode a frameshift mutation disrupting the steroid-binding domain of the AR, rendering these mice insensitive to classical AR signaling (28, 29). These

mice are maintained in a C57BL/6  $A^{w-j}$  (agouti) background with the  $AR^{Tfm}$  allele in repulsion to an *Eda* mutation (tabby) on the wild-type X chromosome, a scheme originally engineered to help distinguish offspring genotype by coat color (28). However, a fitness defect arising from the *Eda* mutation in  $Eda^{-/-} AR^{+/+}$  females or  $Eda^{-}/Y AR^{+}/Y$  males limited the number of such AR-wild-type littermates we could generate. Because of this, and to exclude any effect of the *Eda* mutation on UTI phenotypes, we also analyzed WT C57BL/6  $A^{w-j}$  males ( $AR^{+}/Y Eda^{+}/Y$ ) and females ( $AR^{+/+} Eda^{+/+}$ ).

We compared renal colonization 24 hpi following surgical induction of UTI in the 5 genotypes. As expected,  $AR^{+}/Y Eda^{-}/Y$  (tabby males) and  $AR^{+}/Y Eda^{+}/Y$  (WT males) demonstrated high and statistically equivalent kidney titers 24 hpi, as we had previously observed in WT C57BL/6J males (7). In contrast, females with wild-type AR expression,  $AR^{+/+} Eda^{-/-}$  (tabby females) and  $AR^{+/+} Eda^{+/+}$  (WT females), each displayed low renal bacterial burdens, with most females resolving infection (**Figure 12B**). Both  $AR^{+}/Y Eda^{+}/Y$  WT males and  $AR^{+}/Y Eda^{-}/Y$  tabby males demonstrated significantly higher kidney titers than  $AR^{+/+} Eda^{+/+}$  WT females ( $P=0.0159$  and  $P=0.0238$ , respectively). Importantly, the majority of  $AR^{Tfm}/Y Eda^{+}/Y$  mice (TFM males) resolved renal infection by 24 hpi, with kidney bacterial burdens significantly lower than  $AR^{+}/Y Eda^{+}/Y$  WT males ( $P=0.0043$ ) and  $AR^{+}/Y Eda^{-}/Y$  tabby males ( $P=0.0208$ ) but indistinguishable from  $AR^{+/+} Eda^{+/+}$  WT females ( $P=0.4974$ ). These data specify that androgen receptor activation induces susceptibility to severe upper-tract UTI in both sexes.

## Discussion

In this study, we demonstrate that UPEC establishes intraluminal communities within kidney tubules, arising precipitously in androgenized hosts within a narrow temporal window to

nucleate nascent renal abscesses. These intratubular UPEC communities were apparently protected from phagocytosis, as histology reflected an inability of infiltrating inflammatory cells to cross the fibrotic tubular epithelium surrounding these communities. Finally, we show that androgen receptor activation in either host sex underlies increased susceptibility to pyelonephritis and renal abscess formation, a paradigm with significant potential for translational impact.

Abscesses were focal or multifocal, located adjacent to other, uninvolved segments of renal parenchyma and adopting the pattern of a collecting duct unit. This observation suggests that among UPEC that reach the renal pelvis, only a fraction are ultimately successful in accessing and persisting within selected nephrons. While we often contemplate the process of uropathogen ascension up the urethra or from the ureterovesical junction to the kidney (not specifically tested in C3H mice, given their vesicoureteral reflux), it is equally important to consider the “third ascension” from the renal pelvis and calyces to more proximal areas of the nephron. The virulence mechanisms or host factors which enable this tubular ascension are largely unknown, although some studies have implicated immunologic factors influencing ascension and persistence in the collecting duct (30-32).

Once gaining a foothold, UPEC replicate in the intraluminal space, ultimately infecting an entire collecting duct unit. While infiltrating neutrophils may destroy some infected tubules, successful bacterial colonies are separated from surrounding phagocytes by tubular epithelial fibrosis, with inflammatory cells only being visualized when breaches in the epithelium are evident (**Figure 6**). In other disease models, including ischemic kidney injury, transmigration of neutrophils across the tubule epithelium appears to be unimpaired (33-35). Fruitful future investigations in the present model will illuminate pathogen strategies or host factors (anatomic

features such as the basement membrane (36), or cellular programs leading to fibrosis) that impede neutrophil migration into the abscess community. Importantly, while male C3H/HeN mice are unable to resolve renal infection, their survival indicates that abscess formation successfully restricts the spread of infection, either to nearby unaffected renal parenchyma or systemically into the blood. In contrast, congenic males with diminished innate responses fail to form abscesses and succumb to infection (**Figure 6**). We therefore propose a model whereby ascending abscess development is a double-edged sword: bacteria are contained within the tubule *via* fibrosis, preventing overwhelming systemic disease, but this process creates a privileged site for bacterial replication where surrounding inflammatory cells within the abscess lesion are unable to access the expanding UPEC community.

The UPEC intratubular communities observed within the kidney display remarkable parallels to earlier descriptions of intracellular bacterial communities within bladder epithelium (37-40). Though the microenvironment is presumably quite different in these two niches, UPEC in both these colony types evolves morphologically from a bacillary morphology (**Figure 1**) to dense biofilm collections of coccoid bacteria (**Figure 4**). While the importance of type 1 pili has been detailed extensively during cystitis and for intracellular bladder invasion and replication is (19, 20, 41), relatively few studies have interrogated its role in pyelonephritis. One study demonstrated a role for type 1 pilus-mediated interbacterial binding within the tubule following microinjection of UPEC into the nephron (42). Here we found that type 1 pili were required for ascending intratubular community development, with expression throughout the abscess community (**Figure 7**). This finding suggests that anti-virulence strategies directed at type 1 pili assembly (pilicides (43)) or interbacterial binding (mannosides (44, 45)) may interrupt the development of renal abscesses. Overall, the thematic similarity of UPEC morphology in

bladder and kidney hints that UPEC uses conserved programming to proliferate and to ultimately subvert the innate cellular response through biofilm formation within these niches (46). Such community formation also likely impairs antibiotic action, correlating with a clinical need for prolonged antibiotic therapy in severe pyelonephritis and renal abscess. Even before development of frank abscess (which clinically often requires percutaneous or surgical drainage to accomplish resolution), the fibrotic barrier may limit antibiotic diffusion to the bacterial nidus. Further, occlusion of renal tubules by UPEC would preclude local transit of antibiotics in the urinary space as well as inhibit antibiotic action and penetration into the biofilm community.

Androgen receptor activation clearly induced susceptibility to severe pyelonephritis and abscess development; genetic inhibition of AR signaling attenuated severe UTI. The effect of AR activation with DHT was consistent across both sexes, demonstrating that organizational anatomic differences (including the prostate) do not contribute to this phenotype. Further investigation is required to specify the AR-regulated gene networks that mediate UTI susceptibility in androgenized hosts. The observation of severe UTI in androgenized females offers an alternative, non-surgical approach to bladder inoculation (as female mice can be routinely catheterized) in future studies of severe pyelonephritis and renal abscess formation. We previously saw no appreciable phenotype following estrogen depletion (7), and have ruled out a contribution from peripheral aromatization (**Figure 11**). This is in contrast to a current paradigm, supported by some studies, in which estrogen levels are believed to influence UTI pathogenesis (47-51). Instead, we argue that even modest elevations in circulating testosterone (which often parallel increases in estradiol (52-54)) in male and female patients may have a greater effect on both bladder and kidney susceptibility to infection. We present proof of concept that the course of severe UTI can be altered by AR antagonism, suggesting that

antiandrogen therapy may represent an avenue for adjunctive therapy in treatment-refractory cases of complicated UTI. Flutamide is rarely used in fully androgenized patients because of its relatively low AR affinity, inability to block AR translocation to the nucleus, and elicitation of increased androgen secretion in males (26, 27, 55-57). The efficacy of newer-generation non-steroidal antiandrogens (bicalutamide, enzalutamide, *etc.*) in mitigating UTI severity remains to be explored in preclinical models.

Our findings carry notable relevance to certain human patient populations, suggesting that individuals with elevated androgen levels carry an increased risk of UTI. Certainly, the human population with the highest circulating testosterone, adolescent males (58), have the lowest rates of UTI (12), as differences in urogenital anatomy (compared with women) comprise the key defense against UTI in otherwise healthy men (7). In fact, when these anatomic barriers are compromised or bypassed, epidemiologic data reflect increased morbidity and mortality in men who do develop pyelonephritis and complicated UTI, as compared with women (59-62). The present finding that androgen-mediated UTI receptivity likewise affects females greatly extends the translational implications of our work. Most clinical studies of polycystic ovary syndrome (PCOS), a common hyperandrogenic state in young women, have not specifically ascertained UTI incidence in this population. However, in the few studies in which such data were collected, women with PCOS exhibited increased rates of UTI compared to healthy females (63-65), and UTI incidence decreased in affected women following androgen deprivation therapy (63). In murine models of PCOS, mice overexpressing LH (and consequently testosterone) develop spontaneous pyelonephritis and associated renal damage (66-69). Additionally, an untold number of women without overt hyperandrogenism might be at higher risk for developing UTI because of circulating testosterone levels near the upper limits of the “normal” range, a potential

causative relationship that mandates further study. In a related vein, male infants (*e.g.*, those under 6 months of age) presenting with UTI outnumber their female counterparts, with male UTI rates falling steadily from the neonatal period to late infancy (70-78). This epidemiologic phenomenon closely parallels the postnatal surge in testosterone in male infants that reaches pubertal levels shortly after birth, then steadily wanes to a prepubertal baseline by 6-9 months of age (79-82). We speculate that elevated testosterone levels in male infants are an underappreciated contributor to increased UTI rates in male infants less than 9 months of age. While some male infant UTI has been commonly ascribed to indistinct “urodynamic immaturity,” testosterone activity may actually underlie UTI risk, especially in the two-thirds of such infants (after first febrile UTI) who lack demonstrable vesicoureteral reflux or obstruction (83).

In summary, we present detailed snapshots of the development of ascending UPEC renal abscess, in an emerging model applicable to both host sexes. We further demonstrate that activation of the androgen receptor potentiates severe UTI in male and female hosts. Ongoing work will specify the mechanisms by which AR agonism influences host-pathogen interaction and immune control of UPEC infection in the kidney, illuminating potential avenues to translational intervention in patients with recurrent or severe UTI.

## **Materials and methods**

### *Bacteria*

Uropathogenic *E. coli* strain UTI89 was isolated from the urine of a patient with cystitis (84). The  $\Delta fimH$  deletion strain was generated using a Lambda red recombinase scheme (85) and confirmed by direct sequencing. For infections, bacteria were grown in static Luria-Bertani (LB)



broth for 16 h at 37°C. Cultures were centrifuged for 10 min at  $7,500 \times g$  at 4°C before resuspension in sterile phosphate-buffered saline (PBS) to a final density of  $4 \times 10^8$  CFU/mL.

#### *Surgical and catheterization murine models of UTI*

All animal care and use protocols received prior approval from the Washington University Animal Studies Committee. Experiments were conducted in C3H/HeN (Envigo, Indianapolis, IN), C3H/HeJ (Jackson Laboratories, Bar Harbor, ME), or C57BL/6  $A^{w-J}/A^{w-J}$  (Jackson Laboratories) strains. Mice were housed under constant temperature and humidity, with a 12 h light/dark cycle. Surgical infection of male or female mice was carried out as described previously (7). Mice aged 8-9 wk were anesthetized with inhaled 3% isoflurane, and the abdomen was shaved and sterilized with 2% chlorhexidine solution. A 3-mm vertical, midline incision was made over the bladder through the skin and peritoneum. The bladder was aseptically emptied before injection of 50  $\mu$ L containing  $1-2 \times 10^7$  CFU into the bladder lumen over 10 s. The bladder was allowed to expand for an additional 10 s before the needle was removed, and the peritoneum and skin were closed with sutures. UTI was allowed to proceed from 1 h to 4 wk. For certain female-only experiments as indicated, UTI was initiated by catheter in mice aged 8-9 wk via instillation of 50  $\mu$ L containing  $1-2 \times 10^7$  CFU into the bladder by transurethral catheterization (86-88).

#### *Determination of urine and tissue bacterial loads*

Where indicated, we obtained post-infection, clean-catch urine samples using gentle suprapubic pressure for serial dilution and plating to enumerate CFU/mL urine. At the indicated time points, mice were euthanized via CO<sub>2</sub> asphyxiation, and bladders and kidney pairs were

aseptically removed and homogenized in 1 ml or 0.800 ml sterile PBS, respectively. To ascertain prostate bacterial loads, the male urogenital system was removed *en bloc* and the prostate was micro-dissected under a light dissecting microscope as previously described (89). Briefly, the vas deferens, seminal vesicles, bladder, ureters, prostatic urethra, and adipose tissue were removed from the prostatic tissue before homogenization in 1 ml sterile PBS. We plated serial dilutions of tissue homogenates on LB agar to enumerate bacterial loads. Where indicated, infection in C3H/HeN mice was classified as “chronic” if all urine and endpoint bladder titers contained  $>10^4$  CFU/ml. “Resolved” mice demonstrated endpoint bladder burdens and at least one urine time point  $<10^4$  CFU/ml. Organ homogenates to be used for soluble cytokine analysis were centrifuged at  $15,000 \times g$  for 5 min, and supernatants were stored at  $-80^\circ\text{C}$ .

#### *Tissue histopathology and immunofluorescence*

Infected bladders and kidneys were bisected and fixed in 10% neutral buffered formalin for 24 h. Fixed tissues were embedded in paraffin, sectioned, and stained with H&E or with Gomori trichrome stain. For immunostaining, unstained slides were deparaffinized, washed in PBS, antigen retrieved by boiling in sodium citrate, blocked in 1% BSA, 0.3% Triton-X 100 in PBS for 30 min at room temperature, and incubated with rabbit anti-*E. coli* (E3500-06C, US Biological, Salem, MA) or rabbit anti-gel-purified type 1 pili (90) primary antibodies overnight at  $4^\circ\text{C}$ . After washing in PBS, sections were stained with AlexaFluor 488-conjugated goat anti-rabbit IgG (Life Technologies, Grand Island, NY) secondary antibody and SYTO 61 red fluorescent nucleic acid stain (Molecular Probes, Eugene, OR). Images were acquired on a Zeiss Axio Imager M2 fluorescence microscope.

### *Castration and androgen treatment*

Male mice aged 4 wk were prepared for surgical castration with preoperative subcutaneous injection of buprenorphine (0.05 mg/kg). Males were maintained under inhalation anesthesia with 3% isoflurane via vaporizer and nose cone. Anesthetized mice were positioned supine, and the scrotum was shaved and depilated with Nair (Church & Dwight Co., Ewing, NJ). The operative field was rinsed with sterile water and disinfected with 2% chlorhexidine solution. A 1-cm ventral, midline incision was made in the scrotum, and the skin was retracted to expose the tunica. The tunica was pierced, and the testis and vas deferens were mobilized from the incision. The spermatic cord was clamped and ligated with 4-0 Vicryl above the epididymis, and the testis and vas deferens were removed just below the ligature. The differential vessels and ducts were replaced back into the tunica. This procedure was repeated on the contralateral side. The skin incision was closed with Vetbond (3M Animal Care Products, St. Paul, MN). Where indicated, 60-day continuous release pellets containing 25 mg testosterone, 25 mg DHT, 75 mg flutamide, or placebo (Innovative Research of America, Sarasota, FL) were implanted subcutaneously in sterile fashion at the nape of the neck. DHT pellets were implanted 4 days following castration or sham operation. In females or flutamide-treated males, testosterone, DHT, flutamide, or placebo pellets were implanted at 4 wk of age. All mice were infected at 8-9 wk of age.

### *Serum testosterone analysis*

Venous blood was collected by submandibular puncture using 5-mm steel lancets (MediPoint, Mineola, NY) into Microtainer serum separation tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were allowed to clot for 90 min at room temperature before centrifugation at  $10,000 \times g$ . Serum testosterone was measured by enzyme immunoassay (Immuno-Biological

Laboratories, Minneapolis, MN) at the Ligand Assay and Analysis Core, University of Virginia Center for Research in Reproduction (Charlottesville, VA). Each sample was measured in duplicate and recorded as the mean.

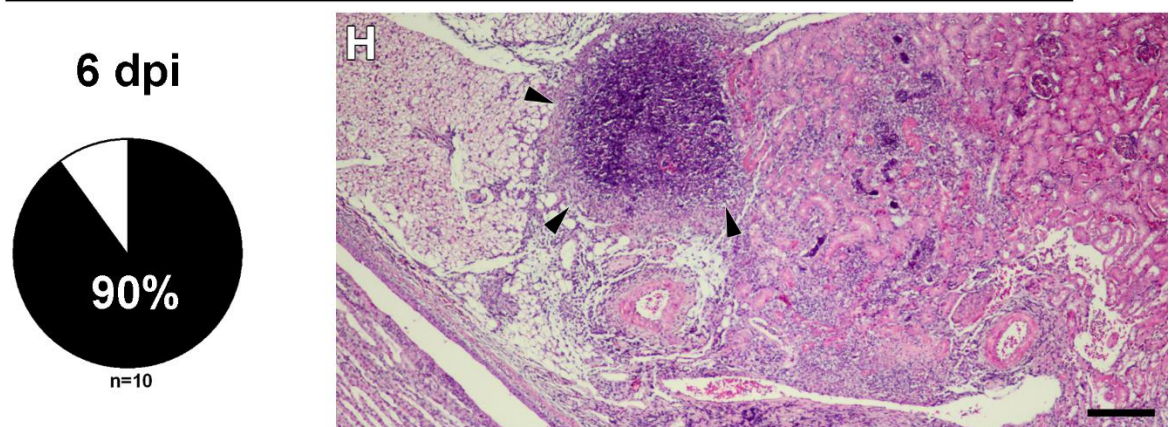
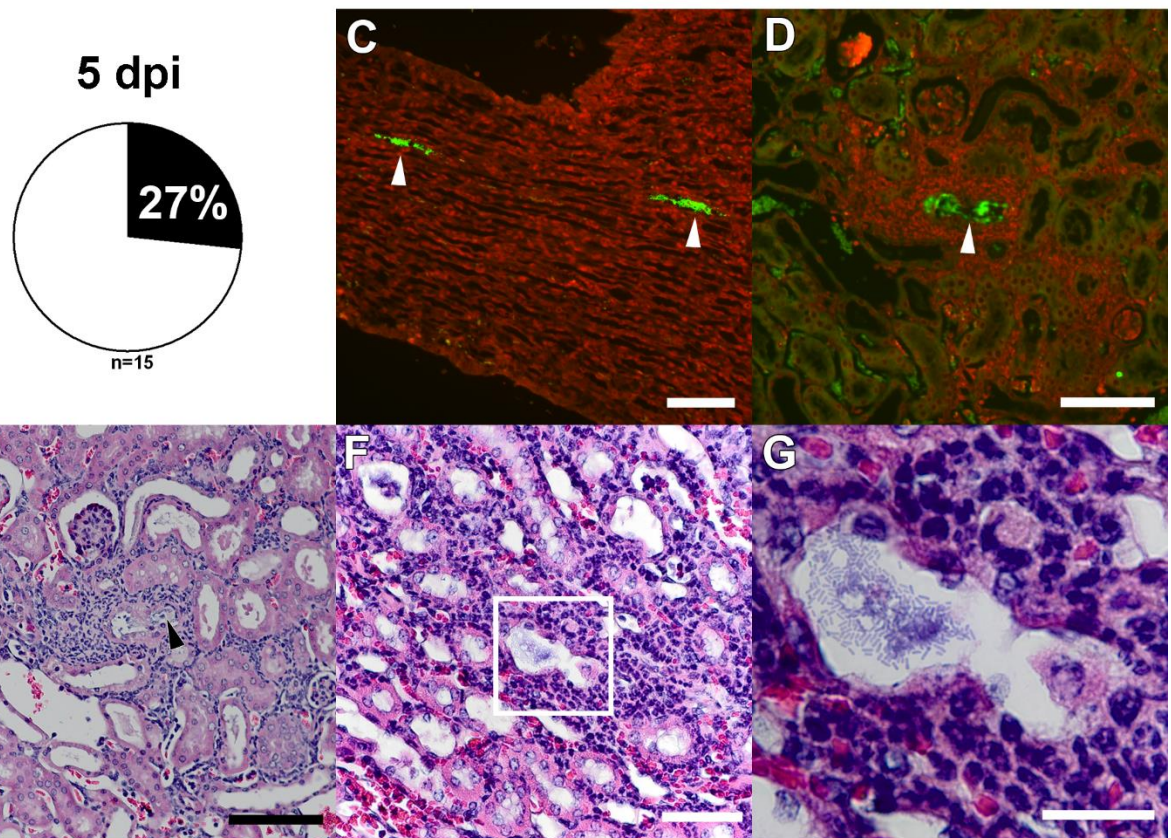
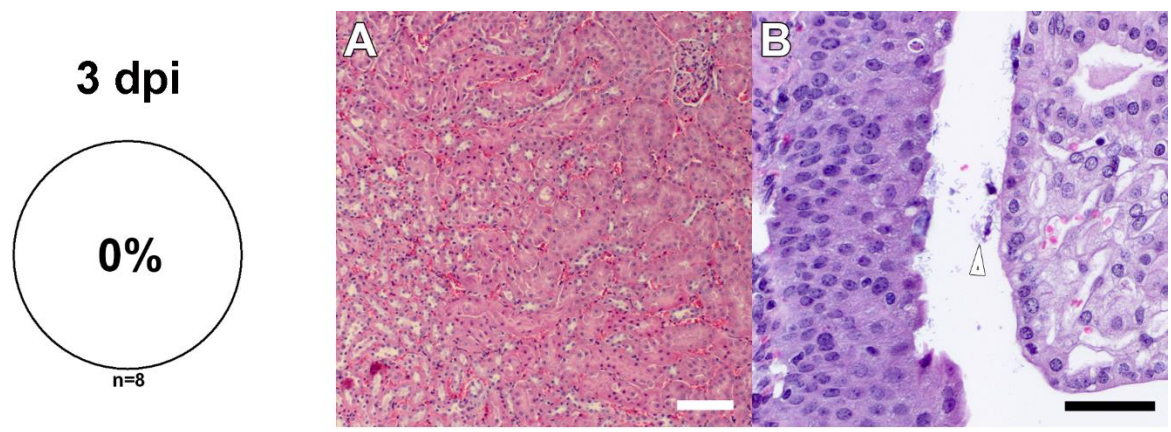
### *Statistics*

Organ bacterial loads and other numerical data were compared by the nonparametric Mann-Whitney U test. Survival analysis was performed with the Mantel-Cox log-rank test. 2x2 comparisons were performed using the Fisher exact test. *P* values <0.05 were considered significant.

## **Acknowledgements**

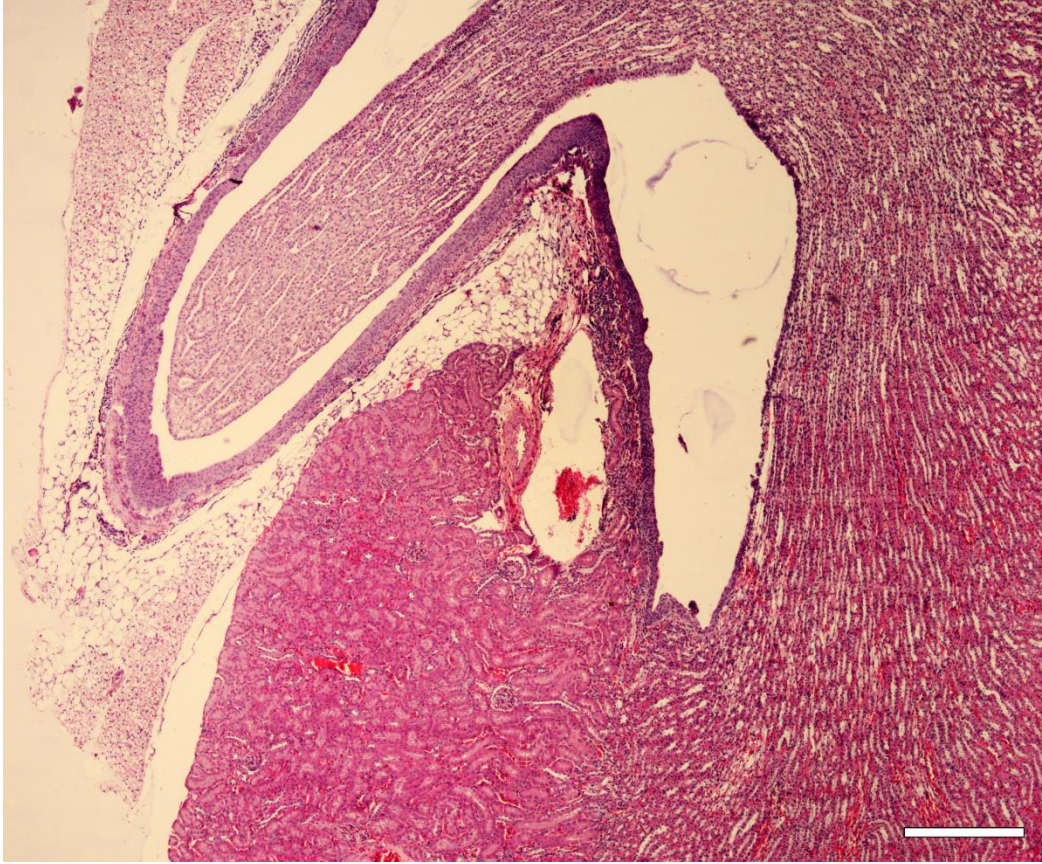
This work was supported by NIH grants P50-DK064540 (and associated supplement), T32-AI007172, and F30-DK104446. We thank Dr. S. Hultgren for the rabbit anti-type 1 pili antiserum, the Elvie L. Taylor Histology Core for histology services, and K. Tiemann and D. Rosen for technical contributions.

## **Figures**



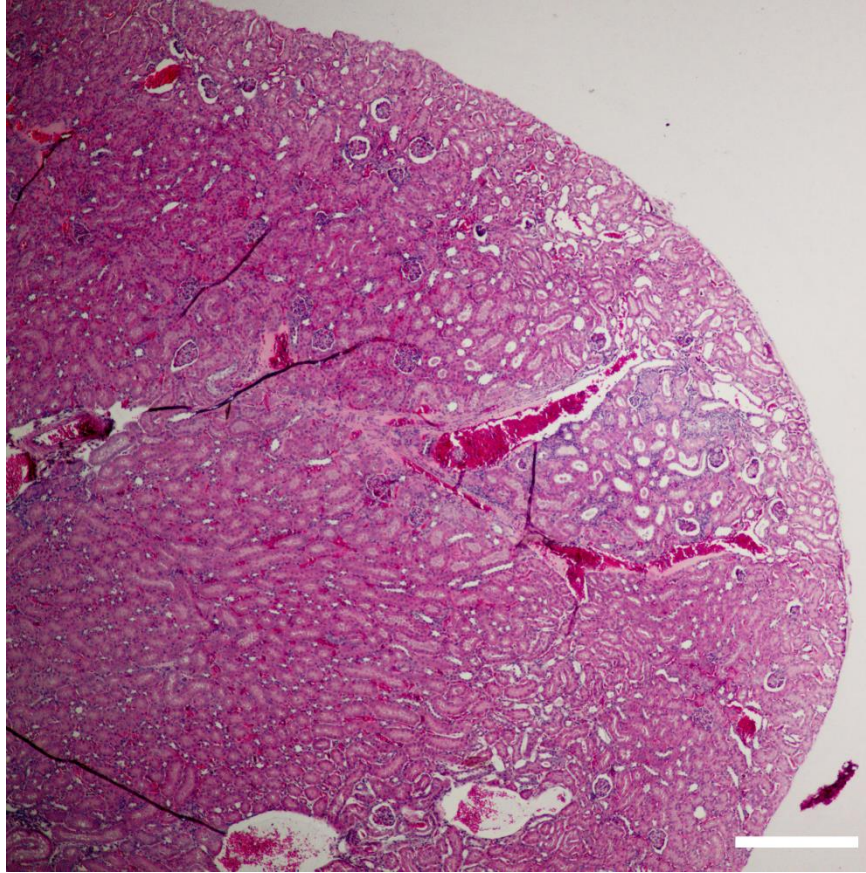
**Figure 1. Timeline of renal abscess formation in C3H/HeN mice.** Pie charts reflect the proportion of male C3H/HeN mice exhibiting gross abscess formation at necropsy at the indicated time points. At 3 dpi, histology demonstrates normal architecture (**A**) and bacteria within the renal pelvis (**B**, black arrowhead). At 5 dpi, immunofluorescence identifies small collections of UPEC (green; white arrowheads) in collecting ducts (**C**) and proximal tubules (**D** and **E** are from adjacent sections); SYTO 61 stains nuclei red. Small numbers of neutrophils surround infected tubules (**E**, **F**), and tubules bear modest collections of bacillary UPEC (inset from **F**, shown in **G**). By 6 dpi, histologic evidence of abscess is fully established, with necrotic lesions, loss of tubular architecture and replacement by neutrophilic infiltrate (**H**, black arrowheads). Scale bars: **A**, 100  $\mu\text{m}$ ; **B**, 50  $\mu\text{m}$ ; **C**, 100  $\mu\text{m}$ ; **D**, 100  $\mu\text{m}$ ; **E**, 100  $\mu\text{m}$ ; **F**, 50  $\mu\text{m}$ ; **G**, 20  $\mu\text{m}$ ; **H**, 200  $\mu\text{m}$ .



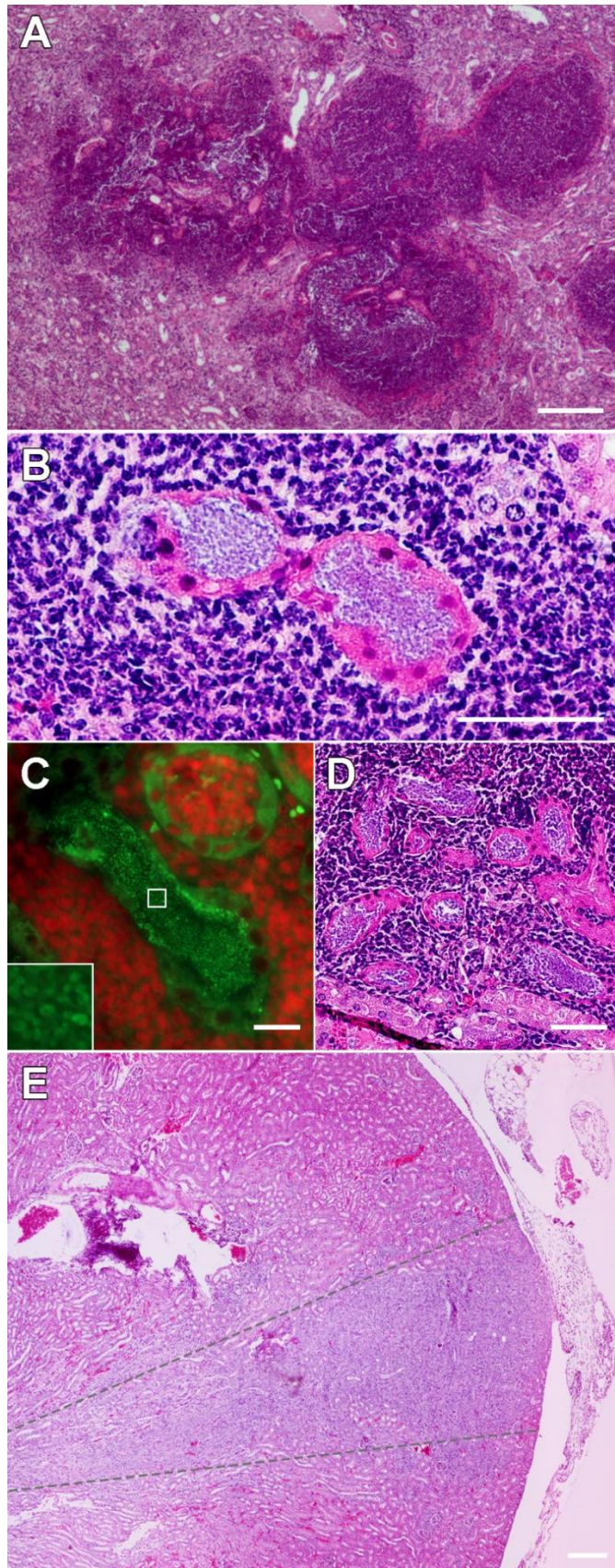


**Figure 2. Male C3H/HeN do not have renal parenchymal involvement 3 dpi.** At 3 dpi in C3H/HeN males, there was no evident inflammation or damage in the renal cortex or medulla. Scale bar, 200  $\mu\text{m}$ .





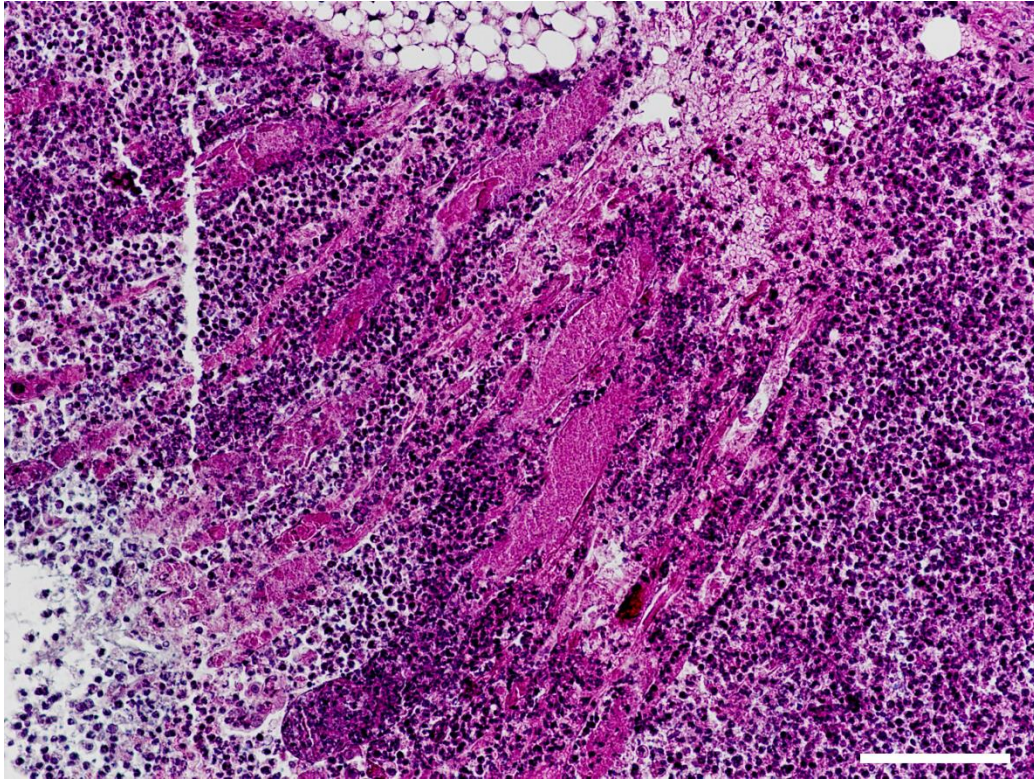
**Figure 3. Male C3H/HeN develop microabscess 5 dpi.** Low-power view (H&E) of infected male C3H/HeN mouse kidney 5 dpi, showing largely preserved tubular architecture and intraluminal bacterial collections. Scale bar, 200  $\mu$ m.



**Figure 4. UPEC establish biofilm-like communities in a protected intraluminal niche.**

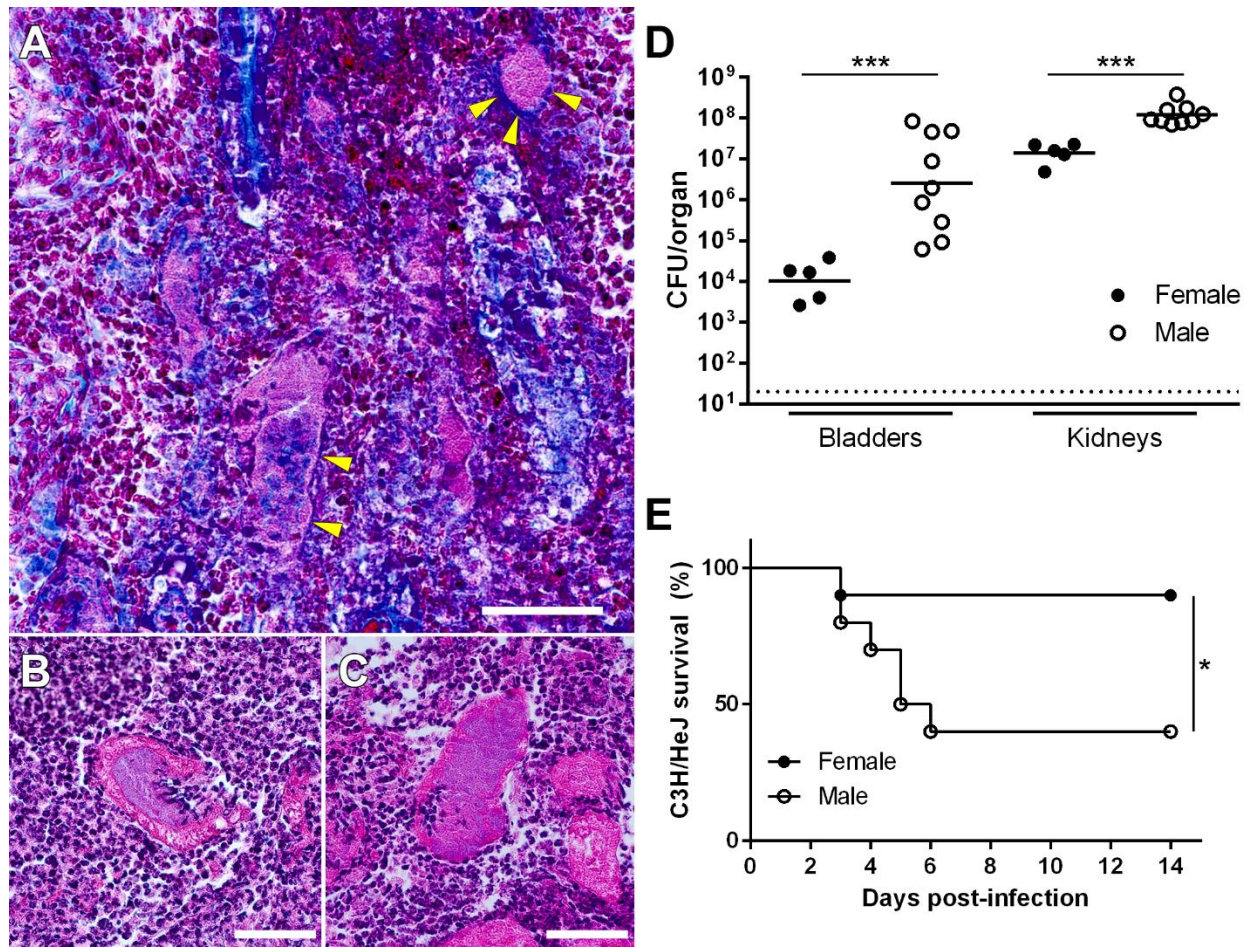
Neutrophilic abscesses were seen throughout the renal parenchyma 2 wpi (**A**). Infected tubules at this time point were filled with coccoid UPEC, as seen by H&E staining (**B**) or immunofluorescence with anti-*E. coli* antibodies (green in **C**, inset shows magnified view; red staining of host nuclei as well as green autofluorescence of tubular epithelium, best seen in upper right, are also shown). In many infected kidneys, multiple infected tubules were identified within a single large abscess lesion (**D**). Overall, the pattern of inflammation followed that of a wedge of nephrons associated with an infected papillary collecting duct (duct of Bellini), generally outlined by the gray dashed lines in (**E**). Scale bars: **A**, 200  $\mu\text{m}$ ; **B**, 50  $\mu\text{m}$ ; **C**, 20  $\mu\text{m}$ ; **D**, 50  $\mu\text{m}$ ; **E**, 200  $\mu\text{m}$ .





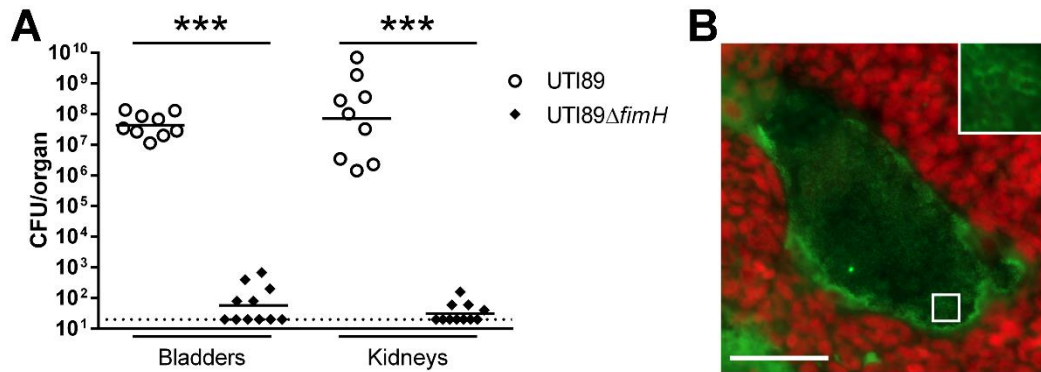
**Figure 5. Female C3H/HeN are susceptible to UPEC intratubular abscess community formation.** Abscess identified 2 wpi in a single female C3H/HeN mouse, demonstrating an H&E appearance identical to abscesses seen in a majority of male C3H/HeN mice. Features include intratubular UPEC communities separated by tubular epithelia from intense neutrophilic infiltrate, which has replaced much of the nearby tubular architecture.

Scale bar, 100  $\mu$ m.

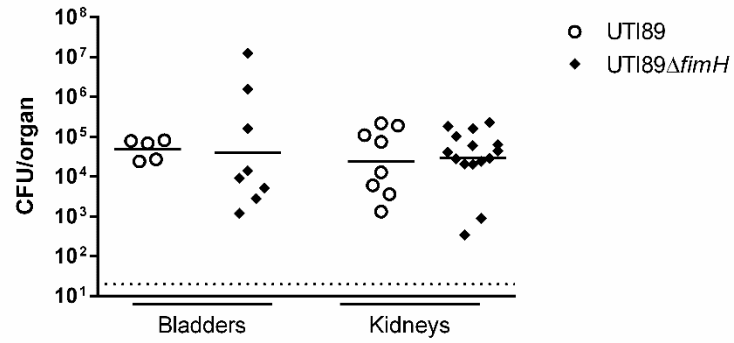


**Figure 6. Fibrotic tubular epithelium restricts infection but protects UPEC from infiltrating phagocytes.** Gomori trichrome staining of kidneys 2 wpi reveals that infected tubules are fibrotic (A, yellow arrowheads). UPEC were undisturbed within infected tubules, except when breaches allowed influx of neutrophils (B, C, H&E). Neutrophil influx was necessary for containment of infection; when the TLR4-deficient C3H/HeJ strain was used, male mice maintained significantly higher bladder and kidney titers than females 2 wpi (D, \*\*\* $P=0.0010$ ; dotted line indicates limit of detection) and were much more likely to succumb to infection (E;  $n=10$  per group, \* $P<0.0274$ ), an outcome not observed in immunocompetent C3H/HeN males. Scale bars, 50  $\mu\text{m}$ .



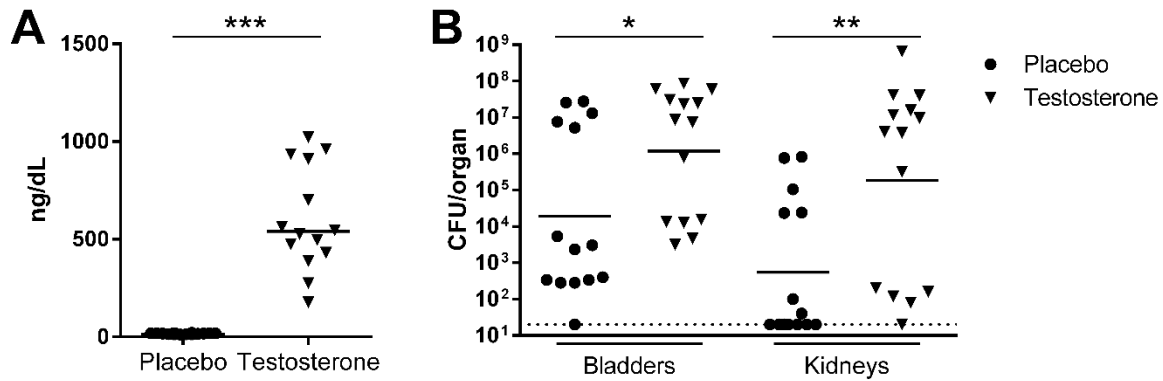


**Figure 7. Type 1 pili are necessary for intratubular community formation.** In C3H/HeN males 2 wpi (**A**), bladder and kidney bacterial loads were significantly lower (with many organs sterile) after infection with UTI89Δ*fimH* (filled diamonds) when compared with WT UTI89 (open circles; \*\*\* $P < 0.0001$ ). Dotted line indicates limit of detection. Immunofluorescence with anti-type 1 pilus antibody demonstrated expression of type 1 pili by coccoid UPEC within the community (**B** and inset; scale bar, 20  $\mu\text{m}$ ). As in **Figure 2**, host nuclei are stained red with SYTO 61, and green autofluorescence of tubular epithelium is present.



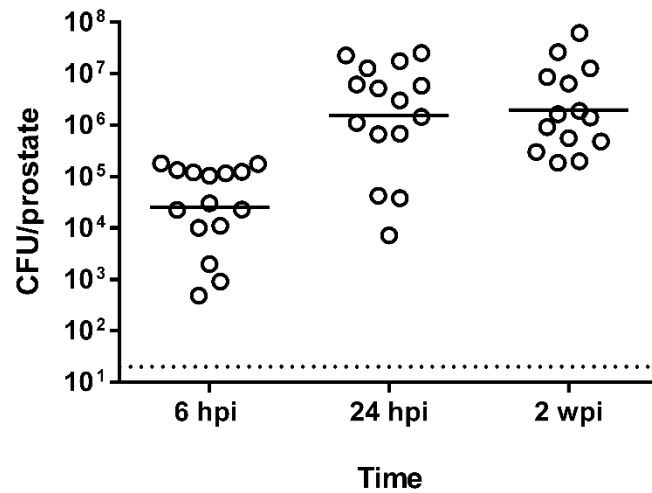
**Figure 8. UTI89Δ*fimH* ascend to the kidneys in numbers equivalent to wild-type 1 hpi.**

Wild-type UTI89 and the *fimH* mutant are recovered at equal numbers from bladder and kidneys 1 h following mini-surgical bladder inoculation of C3H/HeN males. Dotted line indicates limit of detection.

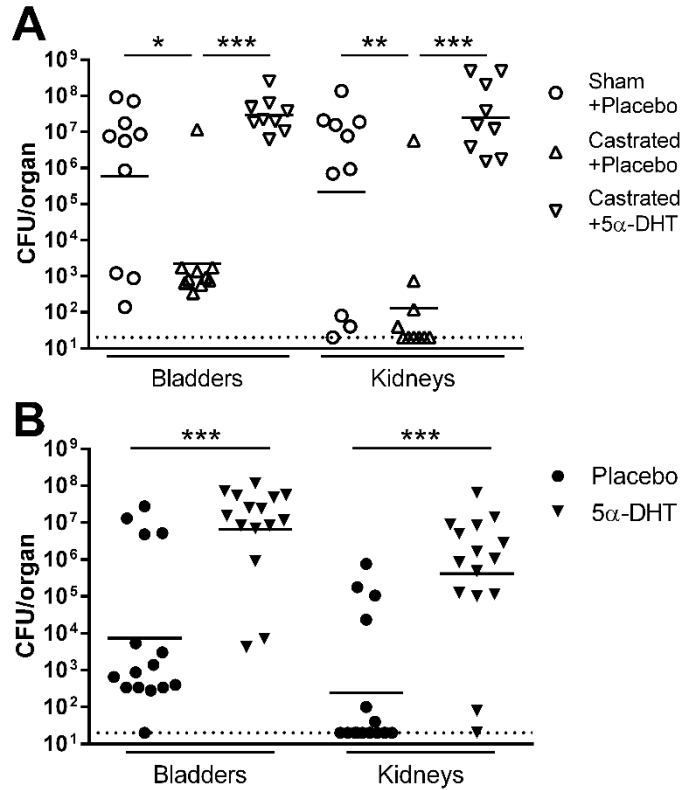


**Figure 9. Androgen exposure aggravates UTI severity in females.** Female C3H/HeN mice were subcutaneously implanted with placebo (filled circles) or long-release testosterone pellets (filled triangles) 4 wk prior to induction of UTI. As anticipated, testosterone pellets increased the serum testosterone to levels physiologically relevant to normal males (**A**, \*\*\* $P < 0.0001$  vs placebo). Organ harvest 2 wpi (**B**) revealed higher bacterial loads in females treated with testosterone, in both the bladders (\* $P = 0.0102$ ) and kidneys (\*\* $P = 0.002$ ). Dotted line indicates limit of detection.





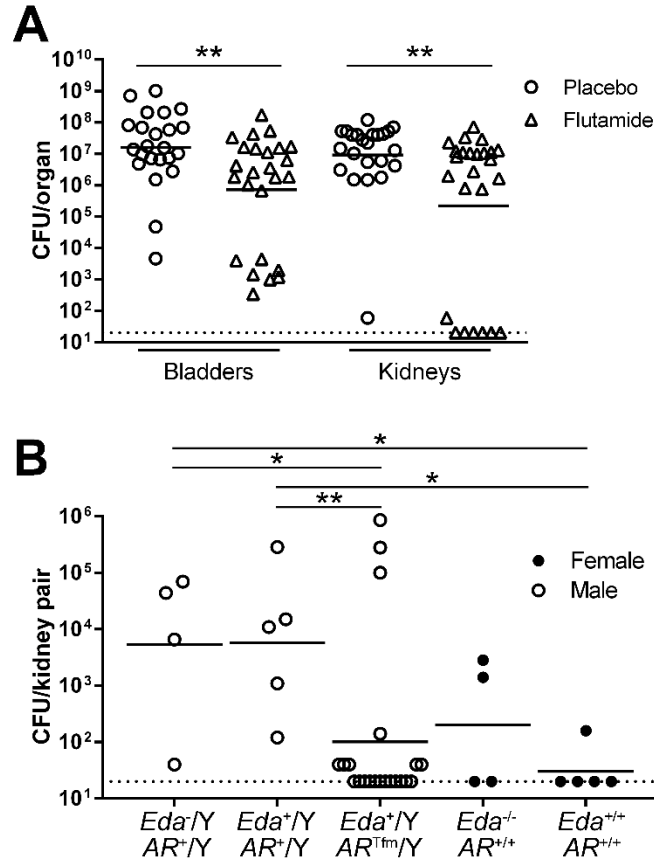
**Figure 10. Surgical induction of cystitis in male C3H/HeN also induces prostatitis.** Mini-surgical inoculation of the bladder in C3H/HeN males results in durable prostate infection with UPEC. Shown are prostate bacterial loads at the indicated time points. Dotted line indicates limit of detection.



**Figure 11. Testosterone influence on UTI severity is not mediated *via* aromatization.**

Bladder and kidney bacterial burdens were measured 2 wpi (**A**) in sham-operated C3H/HeN males treated with placebo pellets (open circles), castrated males treated with placebo pellets (open triangles), and castrated males receiving pellets of 5 $\alpha$ -dihydrotestosterone (DHT), a potent androgen not susceptible to conversion to estrogen via the action of aromatase (inverted triangles). As seen previously, castration was protective against severe UTI (\* $P$ <0.041 in bladder, \*\* $P$ <0.0063 in kidney vs sham-operated). Treatment of castrated males with exogenous DHT reversed this effect (\*\*\* $P$ <0.0001 in both organs vs castrated males receiving placebo); DHT-complemented males exhibited organ bacterial burdens equivalent to sham-operated, placebo-treated males (**A**) and similarly developed gross renal abscess. (**B**) In an analogous way, treatment of C3H/HeN females with DHT was associated with significantly higher bladder and

kidney bacterial burdens 2 wpi (\*\* $P < 0.0001$  vs placebo). Dotted lines indicate limit of detection.



**Figure 12. Androgen receptor activation enhances severity of UTI.** (A) Male C3H/HeN mice were implanted 4 wk prior to infection with a long-release subcutaneous pellet of flutamide (a clinically well-established but imperfect AR antagonist; open triangles) or placebo (open circles). Compared with mice receiving placebo, bladder and kidneys of flutamide-treated mice harbored significantly lower bacterial loads 2 wpi (\*\* $P < 0.01$ ). (B) Renal bacterial loads 2 wpi in five *Eda* and *AR* genotypes comprising phenotypic males (open circles) or females (filled circles). Included strains are tabby males (*AR*<sup>+</sup>/*Y* *Eda*<sup>-</sup>/*Y*), WT agouti males (*AR*<sup>+</sup>/*Y* *Eda*<sup>+</sup>/*Y*), androgen receptor-deficient (TFM) males (*AR*<sup>TFM</sup>/*Y* *Eda*<sup>+</sup>/*Y*), tabby females (*AR*<sup>+/+</sup> *Eda*<sup>-</sup>/*+*), and WT agouti females (*AR*<sup>+/+</sup> *Eda*<sup>+/+</sup>). The renal bacterial loads of functionally AR-deficient (TFM) males were significantly lower than in the *AR*<sup>+</sup> male groups (\* $P < 0.05$ , \*\* $P < 0.01$ ) and were

equivalent to those in the female groups. In fact, the kidneys of most AR-deficient males were sterile 2 wpi (below the limit of detection, indicated by the dotted lines).

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CHAPTER FOUR

RENAL SCAR FORMATION AND CHRONIC KIDNEY DISEASE AFTER ANTIBIOTIC  
TREATMENT OF EXPERIMENTAL PYELONEPHRITIS

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**Abstract**

Urinary tract infection (UTI), primarily caused by uropathogenic *Escherichia coli* (UPEC) is a common, but dangerous disease in infants. Severe kidney infection serves as the primary cause of acquired renal scarring, which can lead to long-term sequelae during adulthood, including hypertension and chronic kidney disease. Advances in our knowledge of UTI have been primarily discovered using a female mouse model. However, female mice show an inherent resistance to severe ascending kidney infection. Consequently, there are currently no preclinical



models to assess the consequences of recovery from pyelonephritis following antibiotic treatment. We recently developed a male model of ascending infection where male C3H/HeN mice develop robust pyelonephritis, renal abscesses, and evidence of fibrosis. Here, we developed a ceftriaxone treatment strategy within our male model to more closely reflect the clinical course of pyelonephritis. A 5-day ceftriaxone regimen administered to male mice at the beginning of abscess development led to resolution of bladder and kidney infection. Despite treatment, some infected mice displayed abscess immediately post-treatment, and a similar fraction of mice developed gross renal scars by 28 days post-treatment. There was dramatic evidence of fibrosis and ongoing inflammation in the scar, despite clearance of any viable bacteria. While renal function was not impaired at 28 days post-treatment, animals developed marked hydronephrosis and evidence of chronic kidney disease by 30 weeks of age. We present the first effective treatment model to study the resolution of severe ascending pyelonephritis. This model offers a platform to study the progression of kidney infection and resulting fibrosis, allowing us to elucidate a more comprehensive understanding of the short and long-term effects of UTI on kidney health.

## **Introduction**

Urinary tract infection is a very common pediatric condition, regularly affecting infants and young children in the first years of life (1-4). Ascension of uropathogens to the kidneys can lead to pyelonephritis, which even with successful antibiotic treatment may carry long-term repercussions for the patient, including the development of renal scarring, hypertension, and eventual progression to end-stage renal disease (5-8). Ascending bacterial infection of the renal parenchyma in humans and mice leads to severe tubulointerstitial inflammation (9-13). This

innate inflammatory response, perhaps more than bacterial processes, may largely underlie renal damage resulting from UTI, and is correlated with loss of functional renal tissue (scarring) and the development of fibrosis (14-17). However, the mechanisms of how and if pyelonephritic scars contribute to chronic kidney disease are unknown (18-20). Further, it is unclear if the location, severity, or timing of renal fibrosis influences progression to chronic kidney disease.

The vague link between infection-related fibrosis and the development of renal disease has been hindered largely by the lack of robust murine models of ascending upper-tract UTI in immunocompetent hosts (9, 12, 13, 21, 22); while cystitis can be induced transurethrally in females of many mouse strains, most are resistant to severe ascending infection of the kidneys (9, 21-23). Previous studies have utilized direct injection of uropathogens into the kidneys, but these models bypass ascension of the ureter (17, 24, 25). Further, existing studies have only examined the generation of inflammation and fibrosis during ongoing, active infection or following spontaneous resolution of infection (9, 12, 13, 26), while human patients would typically receive antibiotic treatment, such as fluoroquinolones or cephalosporins, soon after the onset of symptoms (27).

We previously found using a novel mini-surgical bladder inoculation technique that male C3H/HeN mice, unlike females, develop severe pyelonephritis and renal abscesses following ascending infection, and fail to spontaneously resolve UTI (9). Furthermore, infected males developed fibrosis and progressive renal disease during later stages of infection (9). Here, we extended this new preclinical model of UTI to test the efficacy of antibiotic treatment in severe pyelonephritis and established renal abscesses, and to examine long-term sequelae of infection following antimicrobial treatment.

## Materials and methods

### *Bacteria*

Uropathogenic *E. coli* strain UTI89 was isolated from a patient with cystitis (28). For surgical infections, bacteria were grown statically in Luria-Bertani (LB) broth for 16 h at 37°C. The cultures were centrifuged for 10 min at  $7,500 \times g$  at 4°C before resuspension in sterile phosphate-buffered saline (PBS) to a final density of  $4 \times 10^8$  CFU/ml.

### *Introduction of murine UTI*

A widely used female murine model of cystitis with transurethral inoculation via catheter has been described in methodologic detail (29, 30), but this approach is technically precluded in male animals. A recently developed surgical approach was used to initiate infection in male mice (9). Eight-week-old male C3H/HeN mice (Harlan Laboratories, Indianapolis, IN) were maintained under inhalation anesthesia with 3% isoflurane via vaporizer and nose cone. Anesthetized mice were positioned supine, shaved, and the ventral abdomen was sterilized with 2% chlorhexidine solution. A vertical, midline incision (2-3 mm in length) was made directly overlying the bladder, first through the abdominal skin and then through the peritoneum. The bladder was exposed, aseptically emptied, and punctured with a 30-gauge, 0.5-inch needle adapted to a 1-mL tuberculin syringe containing the bacterial inoculum. Fifty microliters containing  $1-2 \times 10^7$  CFU was introduced to the bladder lumen over 10 s, the bladder was allowed to expand for a further 10 s, and the needle was then withdrawn. The peritoneum and the skin were closed with simple, interrupted sutures, and the animal was awakened in fresh air.

### *Ceftriaxone treatment*

We modified previously described ceftriaxone treatment regimens used in female murine models to clear UTI (31, 32). These dosing regimens result in circulating levels of drug similar to those seen in patients treated with ceftriaxone (31, 32). At 120 h after surgical infection, male C3H/HeN received 125 mg/kg ceftriaxone dissolved in sterile water by intraperitoneal injection. Mock-treated animals received an equivalent volume of PBS. Mice received ip injection of antibiotic or PBS every 12 h for 5 days, receiving 10 doses in total. Bladders and kidneys were aseptically harvested 24 h after the last treatment to allow residual ceftriaxone to clear. For chronic kidney function experiments, UPEC-infected animals that resolved infection spontaneously before treatment or that failed to clear UTI following treatment (urine titers  $>10^4$  CFU/mL) were excluded from analysis.

### *Tissue histopathology*

Infected bladders and kidneys were bisected and fixed in 10% neutral buffered formalin for 24 h. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or with Gomori trichrome stain.

### *Determination of urine and tissue bacterial loads*

Where indicated, we obtained post-infection, clean-catch urine samples using gentle suprapubic pressure for serial dilution and plating to enumerate CFU/mL urine. At the indicated time points, mice were euthanized via CO<sub>2</sub> asphyxiation, and bladders and kidney pairs were aseptically removed and homogenized in 1 ml or 0.800 ml sterile PBS, respectively. We plated

serial dilutions of tissue homogenates on LB agar to enumerate bacterial loads. Where indicated, infection in C3H/HeN mice was classified as “chronic” if all urine and endpoint bladder titers contained  $>10^4$  CFU/ml. “Resolved” mice demonstrated endpoint bladder burdens and at least one urine time point  $<10^4$  CFU/ml. Organ homogenates to be used for soluble cytokine analysis were centrifuged at  $15,000 \times g$  for 5 min, and supernatants were stored at  $-80^\circ\text{C}$ .

#### *Blood and urine chemistry*

Serum or urine was analyzed on the day of blood draw for blood urea nitrogen (BUN), serum creatinine, or urine protein by standard autoanalyzer laboratory methods performed by Department of Comparative Medicine core facility.

#### *Inulin clearances*

Inulin clearances were performed at 28 days post-treatment according to manufacturer instructions (BioPal Inc., Worcester, MA).

#### *Statistics*

Organ bacterial loads and other numerical data were compared by the nonparametric Mann-Whitney U test. 2x2 comparisons were performed using the Fisher exact test.  $P$  values  $<0.05$  were considered significant.

## Results

### *Infection and treatment model.*

Female murine models have been successfully used to interrogate many questions on UTI pathogenesis in the bladder, but females of most mouse rarely develop severe pyelonephritis or renal abscess (9, 21-23). Even females in the C3H/HeN background (recognized to have vesicoureteral reflux (21, 33)) that develop chronic pyelonephritis (without abscess) and are treated with antibiotics maintain normal renal function and do not display evidence of renal scars (22, 34). Because of this, there exist no optimal preclinical models of antibiotic-treated severe pyelonephritis. Thus, we employed our mini-surgical infections of male mice to model the resolution and sequelae of severe pyelonephritis. By 14 days post infection (dpi) with uropathogenic *Escherichia coli* (UPEC) strain UTI89, greater than 90% of surgically infected C3H/HeN males develop gross, bilateral renal abscess (9). In our efforts to model antibiotic treatment, we first attempted multiple ceftriaxone dosing schemes starting 14 dpi in male C3H/HeN mice; these strategies failed to effectively treat the advanced abscesses in kidneys established by that time point (data not shown). Further, we felt it likely that patients would more commonly present earlier in the course of pyelonephritis. We previously demonstrated that abscesses develop rapidly in male C3H/HeN between 5 and 6 dpi, and are fully formed by 7 dpi (**Chapter 3**). Therefore, we next elected to initiate intraperitoneal ceftriaxone (given every 12 hours [q12h] for 5 days) beginning 5 dpi, harvesting organs 24 h after the final dose of antibiotic (*i.e.*, 11 dpi; **Figure 1A**). Mini-surgical bladder inoculation in male C3H/HeN mice resulted in robust bladder (**Figure 1B**) and kidney (**Figure 1C**) infection in both start-of-treatment controls (5 dpi) and mock-treated animals. Ceftriaxone treatment significantly reduced the bladder (**Figure 1B**;  $P<0.0001$ ) and kidney (**Figure 1C**;  $P<0.0001$ ) bacterial burdens compared to

mock-treated animals. Antibiotic-treated mice resolved bacteriuria over the course of treatment (**Figure 1D**). Ceftriaxone-treated mice continued to harbor  $10^2$ - $10^4$  colony-forming units (CFU) of UPEC in their bladders, despite resolving bacteriuria (**Figure 1B and D**); this is consistent with prior reports of UPEC reservoirs residing within bladder tissue following antibiotic treatment (9, 35, 36). The majority of treated mice completely resolved kidney infection following antibiotic treatment (**Figure 1C**). Other treatment regimens beginning 5 dpi, including increased ceftriaxone duration, dose, or frequency, did not further affect the organ bacterial burden (data not shown) compared to this 5-day, q12h regimen.

Among mice sacrificed at the start of treatment (5 dpi), all of which had high kidney bacterial burdens (**Figure 1B**), a minority (4 of 15, 27%) demonstrated gross abscess (**Figure 2A**), matching our previous report at the same time point (9). All (10 of 10) infected, mock-treated males displayed gross renal abscess formation by harvest 11 dpi (24 h post treatment completion; **Figure 2B**). Thus, abscess development continued to progress during this six-day interval in the absence of antibiotic treatment. However, ceftriaxone-treated mice displayed gross abscess frequency 11 dpi (4 of 14, 29%) that was equivalent to 5-dpi start-of-treatment controls (**Figure 2C**). As noted above, these ceftriaxone-treated mice with evident abscess exhibited kidney bacterial burdens at or near the limit of detection (**Figure 1C**), and did not have ongoing bacteriuria (**Figure 1D**). These data suggest that ceftriaxone treatment arrested renal abscess development and neutralized the burgeoning UPEC population within the renal parenchyma. As expected, control mice (mock-infected with PBS and treated with ceftriaxone) displayed healthy kidney architecture 24 h post treatment (**Figure 2D**).

### *Convalescent outcomes in treated pyelonephritis.*

While the majority of CRO-treated mice demonstrated sterile kidney titers 11 dpi, a small proportion maintained low-titer UPEC burdens. Further, it was unclear if UPEC remaining in the bladder post treatment would reemerge to continue infection. To specify outcomes of treated pyelonephritis, we treated mice with CRO (5 days, q12h) beginning 5 dpi and quantified organ bacterial burdens 4 wk post-treatment. All CRO-treated mice resolved renal and bladder infection (**Figure 3A**;  $P=0.0007$  and  $P=0.0003$ , respectively, vs mock-treated controls). No CRO-treated mice displayed urine UPEC titers  $>10^4$  CFU/mL after biweekly sampling, but a small fraction had low-level colonization of the bladder (**Figure 3A**). Remarkably, gross renal scars were found in several treated mice at necropsy 4 wk post infection (**Figure 3B**, arrowheads). Affected kidneys demonstrated broad-based, “U”-shaped cortical scarring with retraction of the renal parenchyma, matching the pathological descriptions of human pyelonephritic scars (37). The fraction of mice displaying grossly visible renal scars 4 wk following CRO treatment was similar to the fraction of mice demonstrating abscess at either start of treatment (5 dpi) or 1 day post treatment (11 dpi) (**Figure 3C**). Collectively, these data suggest that the tissue destruction associated with microscopic abscess formation is pathologically associated with the ultimate development of renal scars.

### *Ceftriaxone treatment restores renal function at 1 month post treatment.*

Individuals that develop acute pyelonephritis typically restore baseline renal function following resolution of infection, and negative sequelae of resolved infection are not typically seen until later in life (5-8, 38). We thus examined glomerular filtration rate (GFR) by inulin clearance at 4 wk following treatment in mock-infected, ceftriaxone-treated mice; UPEC-



infected, mock-treated mice; and UPEC-infected, ceftriaxone-treated mice (**Figure 4A**). Not surprisingly, chronically infected and untreated C3H/HeN males exhibited impaired renal function ( $P=0.1143$ ). Treatment with ceftriaxone normalized GFR in UPEC-infected mice to levels equivalent to mock-infected, ceftriaxone-treated controls. Histopathologic analysis of Gomori trichrome-stained kidney sections from ceftriaxone-treated mice that resolved infection and lacked renal scars showed no increase in interstitial fibrosis compared to mock-infected, ceftriaxone-treated controls, and had renal architecture similar to naïve, mock-treated animals (**Figure 4B, C**). However, we found a consistent increase in glomerulosclerosis and periglomerular fibrosis in UTI89-infected, ceftriaxone-treated animals (**Figure 4C**).

*Renal scars, despite resolution of infection, harbor progressive inflammation.*

Histopathological analysis of UPEC-infected, ceftriaxone-treated kidney sections by Gomori trichrome staining revealed extensive cortical scars, with collagen deposition extending from the renal capsule to the medulla (**Figure 5A**). Matching our gross observations, these cortical scars appeared microscopically as strictures on the kidney. The renal capsule was dramatically thickened overlying the scar (**Figure 5B, C**, black arrowheads). Fibrosis in these scars followed patterns similar to those observed at earlier stages of abscess development 5 dpi and 11 dpi in infected, ceftriaxone-treated mice (see **Figure 2**). No scars were observed in mock-infected, ceftriaxone-treated animals. More striking was the presence of a cellular infiltrate within the scar (**Figure 5C, D**), despite all tested animals in this group resolving renal infection (**Figure 3A**) and exhibiting sterile urine cultures. Collections of inflammatory cells (primarily lymphocytes) and fibroblasts were embedded within the area of fibrosis (**Figure 5D**). These data

suggest that the development and maturation of renal scars is an active process, even following successful treatment of infection with antibiotics.

*Pyelonephritic scarring leads to chronic kidney disease.*

Children who develop renal scars following UTI are frequently followed into adulthood, when signs of chronic kidney disease may manifest (5-8). Therefore, we surgically infected male C3H/HeN mice with either PBS (mock) or UTI89 and treated with ceftriaxone before aging to 30 weeks of age (*i.e.*, 5 months post treatment). Infected and successfully treated mice demonstrated slightly higher serum creatinine (**Figure 6A**), BUN (**Figure 6B**), and urine protein (**Figure 6C**) at this time interval, but comparisons to mock-infected mice were not statistically significant. However, one UPEC-infected mouse that developed bilateral scars displayed extremely high serum creatinine and BUN, as well as urine protein (**Figure 6A-C**), suggesting it had developed chronic kidney disease. No other UPEC-infected mice demonstrated gross scars in this experiment.

Surprisingly, we found evidence of gross hydronephrosis in most infected males (**Figure 7A**). These animals had dilated ureters, and expansion of the renal pelvis was evident on bisection of the kidneys. Histopathology confirmed these findings, with infected animals displaying enlarged, dilated renal pelvis and calyces, flattening of the pelvic epithelia, atrophy and thinning of the renal cortex, and expanded ureters (**Figure 7B**). These features were notably absent in mock-infected animals (**Figure 7C**). While the frequency of gross scars was lower in this chronic experiment, fibrotic scars were evident microscopically in UPEC-infected mice, but not mock-infected controls (**Figure 7D**). These scars resembled those seen at 38 dpi (**Figure 5**),

with areas of fibrosis and infiltrating inflammatory cells, and were commonly seen surrounding the renal pelvis (**Figure 7D**).

## **Discussion**

Here, we developed a novel antibiotic treatment model to study the resolution and sequelae of severe upper-tract UTI. To do so, we took advantage of the surgical UTI model allowing infection of male C3H/HeN mice, which develop nearly 100% penetrant renal abscess following bladder inoculation with UPEC. While antibiotic treatment was not successful at later time points (presumably because of progressed abscess, and consistent with clinical experience in patients with established abscesses), ceftriaxone treatment at 5 dpi resulted in resolution of infection. Surprisingly, a minority of infected, treated animals developed renal scars by 4 wk post-treatment. These scars displayed ongoing inflammation despite infection resolution, and animals that illustrated scars also demonstrated initial signs of chronic renal disease.

Our preclinical model of renal scarring following treatment of ascending UTI fills a substantial gap in the field. No previously published reports examine outcomes in preclinical models following antibiotic resolution of ascending infection. Instead, studies have been limited to direct injection to the renal medulla (17, 24, 25) or examining renal damage following spontaneous resolution or during active infection (9, 12, 13, 26). In part, progress in this arena has been hampered by the inherent resistance of females in most mouse strains to pyelonephritis (9, 22, 23, 39, 40). Our recent development of a male surgical model of UTI allowed us to take advantage of male C3H susceptibility to pyelonephritis to probe questions on upper-tract UTI pathogenesis (9). Here, we present a preclinical model using our male surgical technique which more closely recapitulates the progression and resolution of human disease where mice develop

ascending pyelonephritis, infection progresses to the beginning of abscess development, and animals receive ceftriaxone.

Roughly one third of infected male C3H/HeN mice exhibited gross abscess at 5 dpi or demonstrated sterile abscess post-treatment. Presumably, those mice that developed abscess with associated necrosis of the renal parenchyma before and/or during ceftriaxone treatment developed a scar in the area of resolved pyelonephritic abscess by 1 mpt, as a third of treated mice demonstrated a renal scar at this time point. However, we currently have no data definitively linking the anatomic locations of developing abscess and ultimate renal scars. Post-treatment abscesses demonstrated inflammation and tissue destruction similar to descriptions of abscess 5 dpi (**Figure 2**, and **Chapter 3**), and inflammation persisted in the renal scar at later time points post treatment (**Figure 5**), consistent with reports from human pathology (41). Thus, we predict that the intense inflammation found in abscesses continues to evolve into the ongoing inflammatory processes present within the renal scar. Further, we predict that the crux of scar development lies in the inflammatory processes affecting the surrounding renal parenchyma. Indeed, anti-inflammatory treatment modalities may be promising routes to circumvent the development of chronic kidney disease, in conjunction or following antimicrobial therapy, to lessen the degree of inflammation and/or impact of inflammatory modulators released from pyelonephritic scars (18, 26, 42, 43).

Only a small percentage of humans presenting with upper-tract UTI develop renal scars following resolution of acute infection; it is unclear why some individuals develop these scars while others do not (38, 44, 45). Estimates of the risk of developing renal scarring after pyelonephritis in children vary, but range between 8% and 40%, with a meta-analysis approximating that 15% demonstrated evidence of scarring on follow up (38, 46). Our model of

pyelonephritic scarring recapitulates this proportion, with 27% of mice developing renal scars. Our previous data demonstrated that a window of 12 to 24 h (at days 5-6 post infection) could make the difference between UPEC infection of the renal parenchyma with abscess formation or limitation to the renal pelvis and calyces (**Chapter 3**). Clinical studies have shown that early and aggressive antibiotic treatment minimizes the risk of renal scar formation (17, 47, 48); our studies reinforce this point, suggesting that minor delays in the start of antimicrobial treatment could substantially influence whether permanent renal damage occurs or if pyelonephritis resolves without complication.

To our knowledge, there has been no demonstrated paradigm of UTI causing the development of hydronephrosis. Several early studies did speculate that infection may cause an increase in VUR, although the evidence for either cystitis promoting VUR or having no effect on reflux is relatively weak (49-53). Conversely, individuals with hydronephrosis have increased risk for UTI because of concurrent urodynamic abnormalities such as vesicoureteral reflux (VUR) or urinary obstruction. The hydronephrosis evident long after antibiotic treatment of infected mice was bilateral (**Figure 7**), suggesting augmented bilateral VUR, obstruction below the bladder trigone (*e.g.*, in the prostatic urethra), or bilateral defect in the ureterovesical junction (the last of which seems unlikely). Further, treated males did not display evidence of hydronephrosis at 4 weeks post-treatment, necessitating some disease progression between then and 30 weeks of age when hydronephrosis was observed. While upper-tract UTI certainly raises kidney damage, bladder and/or ureter remodeling following UTI may represent an under recognized complication which contributes to long-term sequelae. This may prove to be a vicious cycle in patients with urodynamic abnormalities, whom are already predisposed to UTI, but whose urodynamics may regress with every additional UTI.

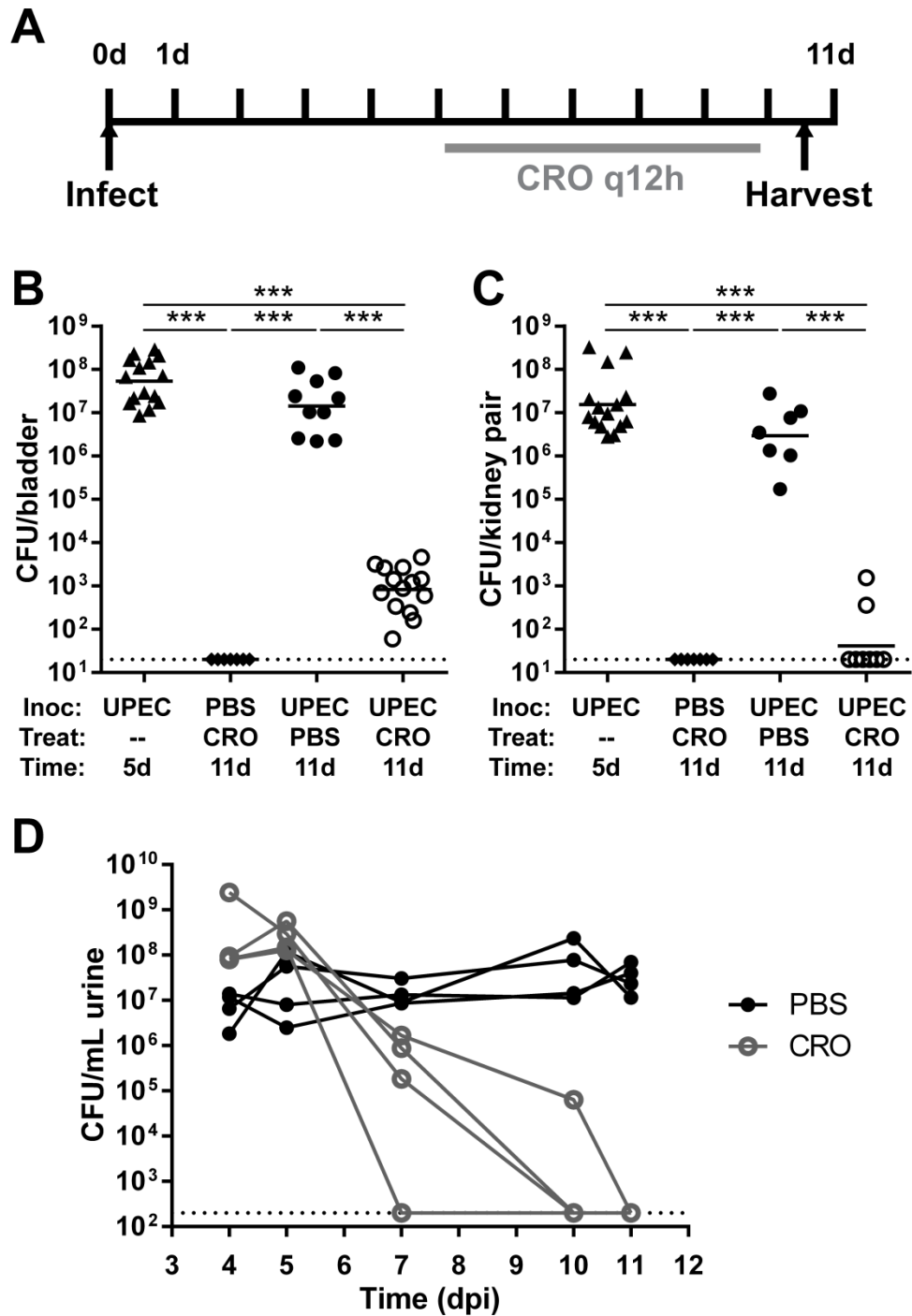
Our initial experiments looking into the development of chronic kidney disease following pyelonephritis produced promising, albeit inconclusive results. Overall, there was no difference in markers of chronic kidney disease between mock- and UTI89-infected animals that received ceftriaxone treatment. However, as noted above, these infection and treatment conditions resulted in only a minority of animals developing gross renal scars, but the single animal that did develop severe pyelonephritic scars had biochemical evidence of renal failure. More penetrant or extensive scar formation may be required to concretely determine whether scar formation associates strongly with chronic kidney disease. This could be potentially overcome by infecting with a greater inoculum or initiating treatment at 6 or 7 dpi, when a larger majority of C3H/HeN males have established gross abscess (9). Alternatively, most murine models of chronic kidney disease require unilateral nephrectomy with injury or insult to the remaining kidney to induce renal failure (54-57), and a similar unilateral or partial nephrectomy procedure could be considered before infection and treatment in our model. Measurement of blood pressure will help to correlate renal scars in mice with hypertension, which is more common as a sequela in human pyelonephritis than CKD. Continued work along these lines will provide further evidence to define the relationship between pyelonephritic scarring and chronic kidney disease.

In this report, we unveil a novel preclinical treatment model to study complications arising from severe UTI. Susceptible hosts develop abscess following ascending UPEC infection, and ultimately develop renal scars (which are not entirely quiescent) after successful antibiotic treatment. This model promises to address the relationship between pyelonephritic scarring and chronic kidney disease, as well as to uncover inflammatory factors and other host pathways responsible for continued renal damage following infection resolution.

**Acknowledgements**

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Figures

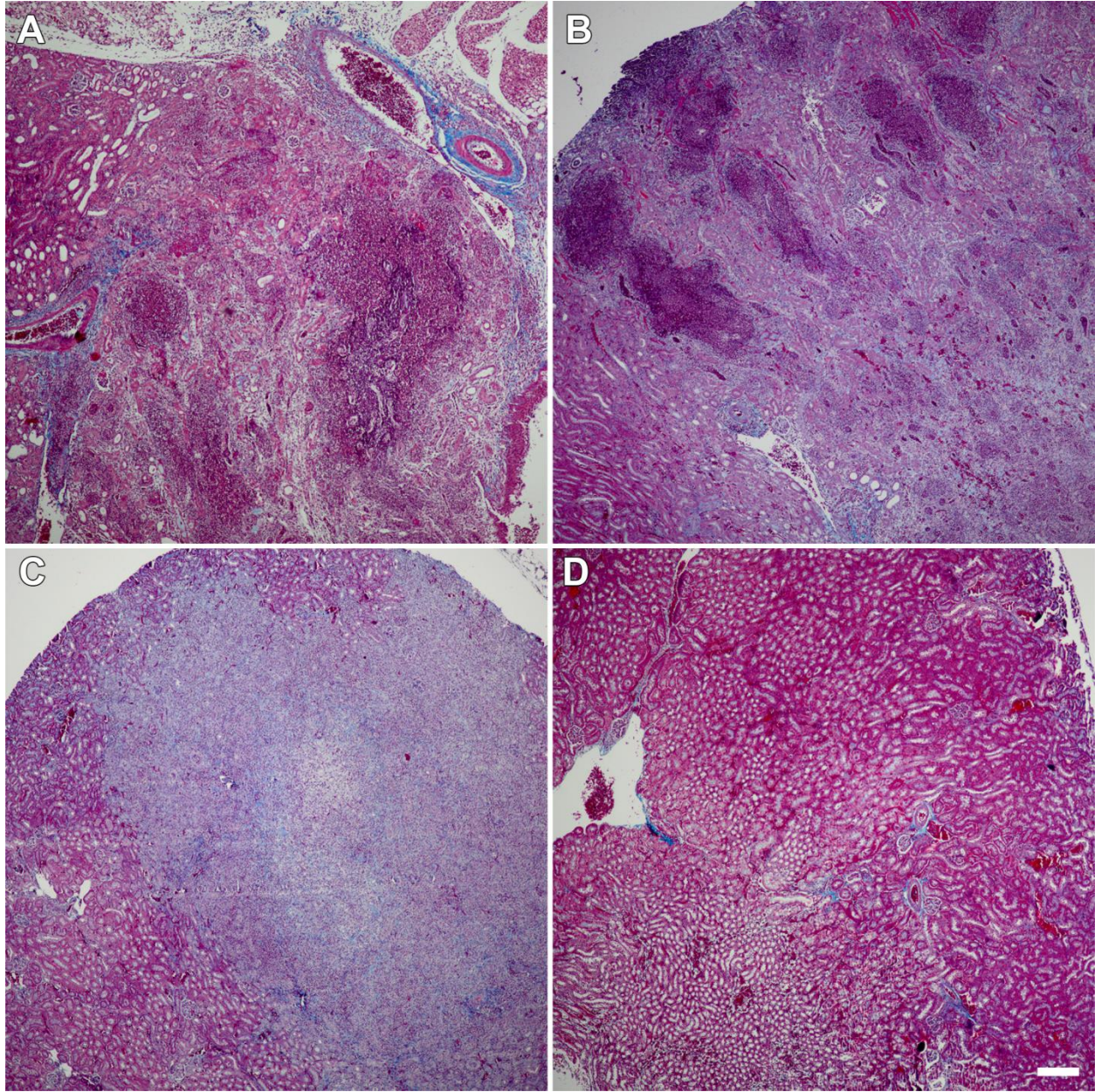


**Figure 1. Ceftriaxone treatment eliminates renal bacterial burden in male C3H/HeN. (A)**

Male C3H/HeN mice were surgically infected with UTI89 or PBS and then treated with ceftriaxone or PBS starting 5 dpi. Bladders (**B**) or kidneys (**C**) were aseptically homogenized



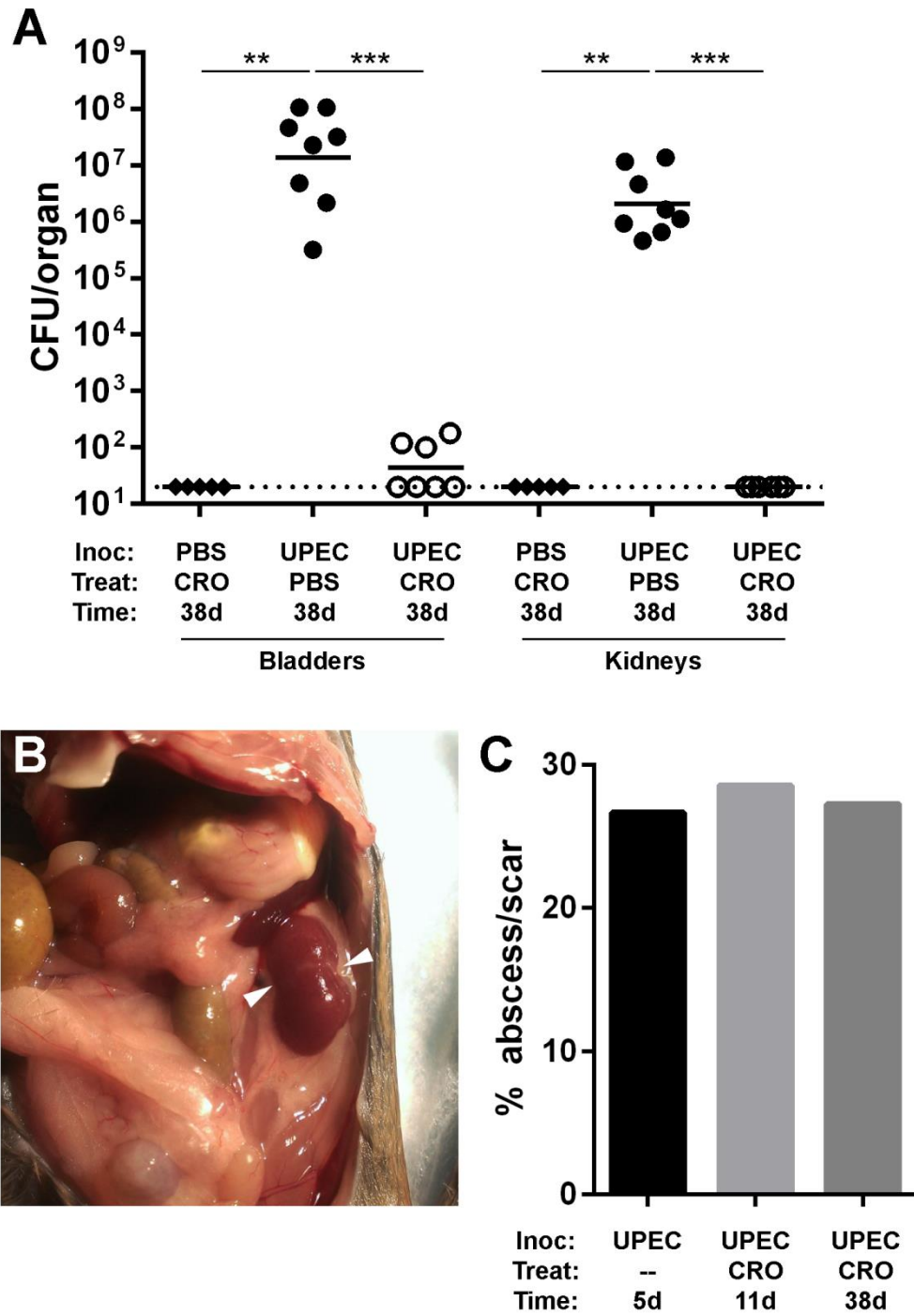
and plated to enumerate CFU 24 h after the last ceftriaxone injection. 5 dpi start-of-treatment controls (triangles) were compared to mock-infected (diamonds), mock-treated (filled circles), or ceftriaxone-treated (open circles) C3H/HeN. Data were compiled from three independent experiments. **(D)** Male C3H/HeN were surgically infected with UTI89 and mock- (black circles) or ceftriaxone-treated (grey circles) 5 dpi. Urine bacterial titers were followed during antibiotic treatment. Solid lines connect corresponding urine time points from each individual mouse. Dashed lines in all panels represent the limit of detection. Bars depict the geometric mean. (\*\*\*)  $P<0.001$ )



**Figure 2. Ceftriaxone treated mice display renal abscess 11 dpi despite resolution of infection.** (A) Male C3H/HeN were mock-infected with PBS and treated with ceftriaxone at 5 dpi. Gomori trichrome staining reveals normal kidney architecture without the presence of abscess 1 day after treatment. (B) All UTI89-infected C3H/HeN receiving PBS (mock) treatment displayed abscess 1 day post treatment. (C) A small fraction (27%) of start-of-treatment controls sacrificed 5 dpi after surgical infection with UTI89 demonstrated renal

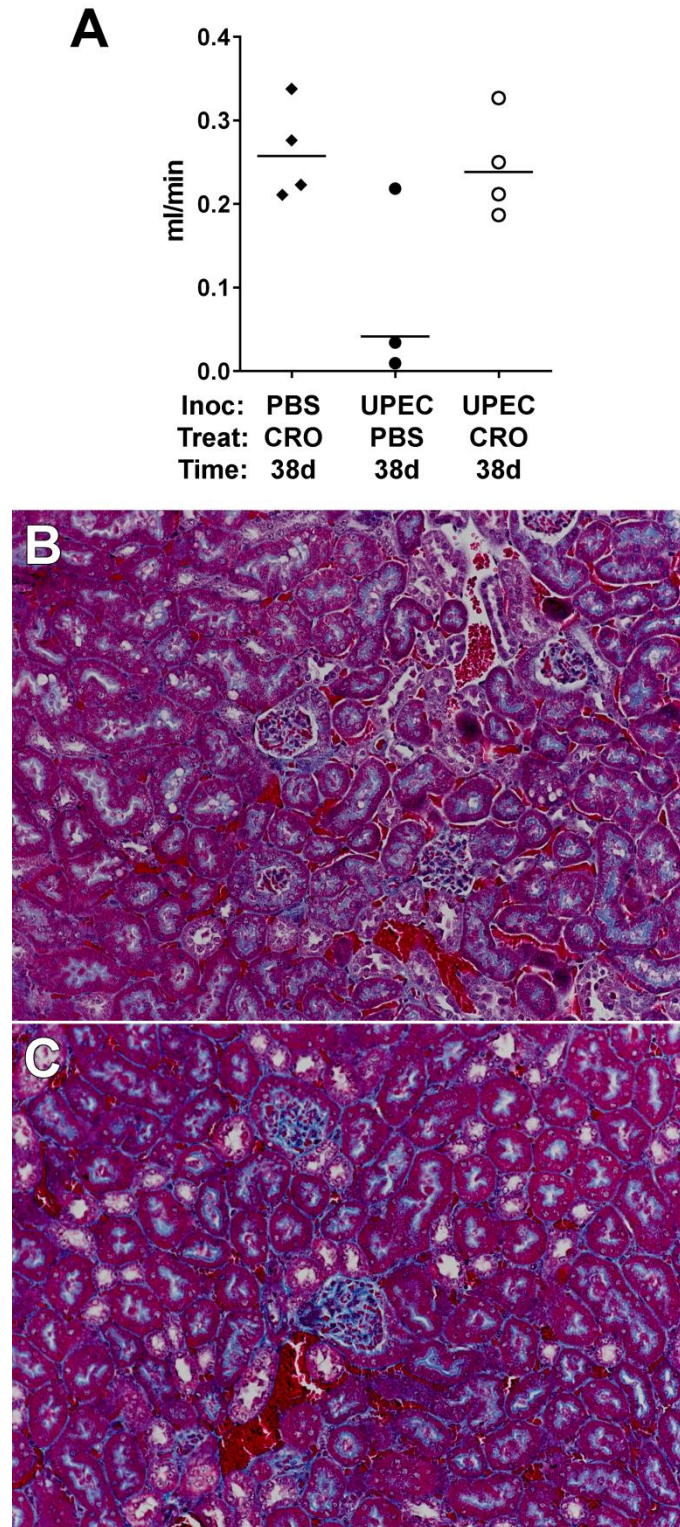
abscess. **(D)** A minority (29%) of UTI89-infected, ceftriaxone treated male mice displayed abscess by Gomori trichrome staining 1 day post treatment, despite resolution of renal infection **(Figure 1)**.





**Figure 3. Ceftriaxone treated mice clear infection and develop gross renal scars by 28 days post-treatment.** Male C3H/HeN were surgically infected with PBS or UTI89 and then treated with PBS or ceftriaxone. (A) Bladders and kidneys were aseptically harvested, homogenized,

and CFU enumerated at 38 dpi (28 d post treatment). Ceftriaxone treatment significantly reduced bacterial burden and resulted in sterile kidney titers 38 dpi (\*\*  $P<0.01$ , \*\*\*  $P<0.001$ ). **(B)** Following autopsy, gross renal scars (white arrowheads) were observed in 27% of UPEC-infected, ceftriaxone treated mice 38 dpi. A representative image of a scarred left kidney is shown. **(C)** The percentage of animals developing abscess 5 dpi, 11 dpi following ceftriaxone dosing (24 h post-treatment), or developing renal scars at 38 dpi (28 d post treatment) is equivalent.

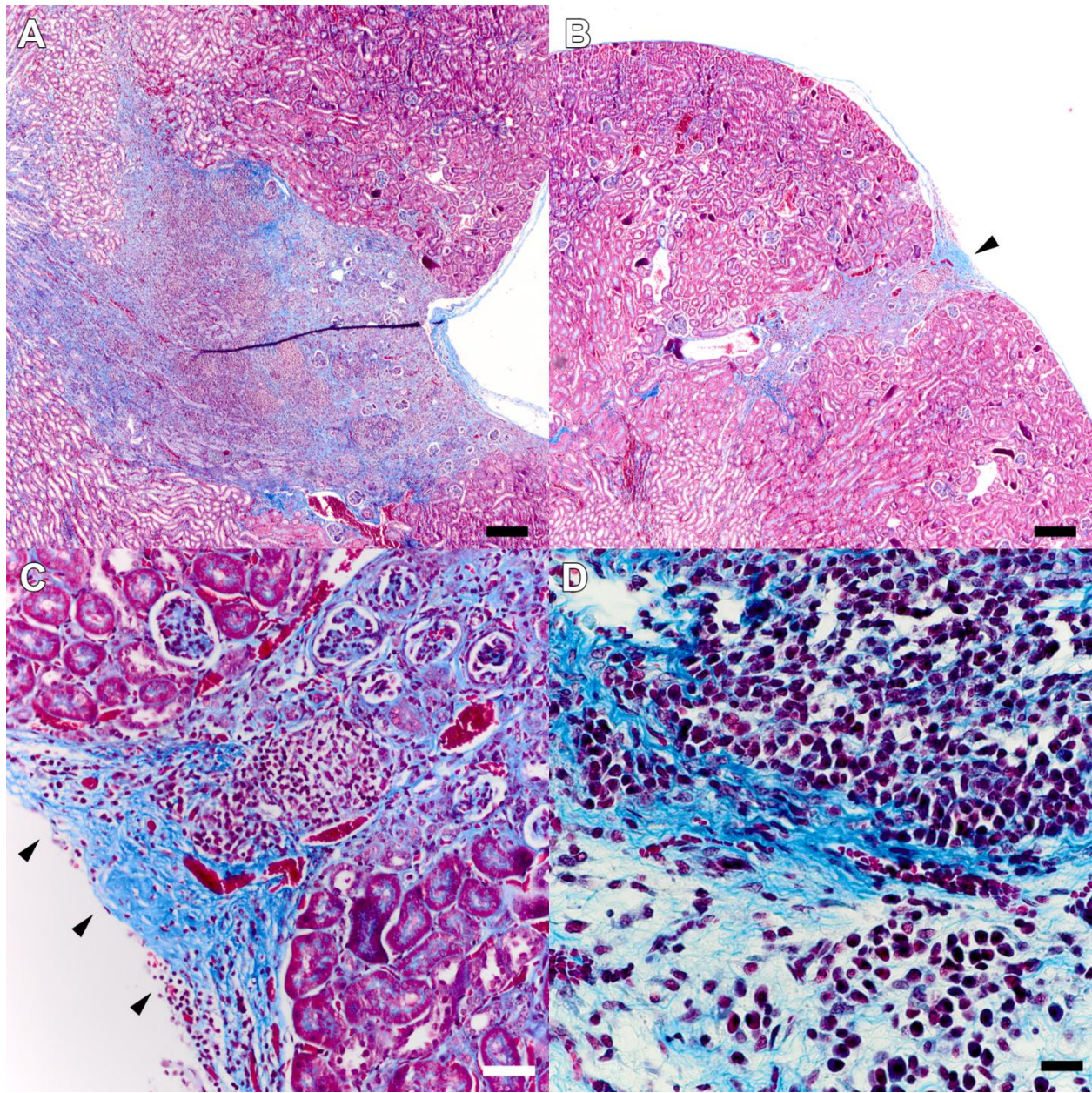


**Figure 4. Treated mice demonstrate healthy renal function 28 days post treatment.**

Glomerular filtration rate was measured by inulin clearance at 38 dpi (28 d post treatment) in

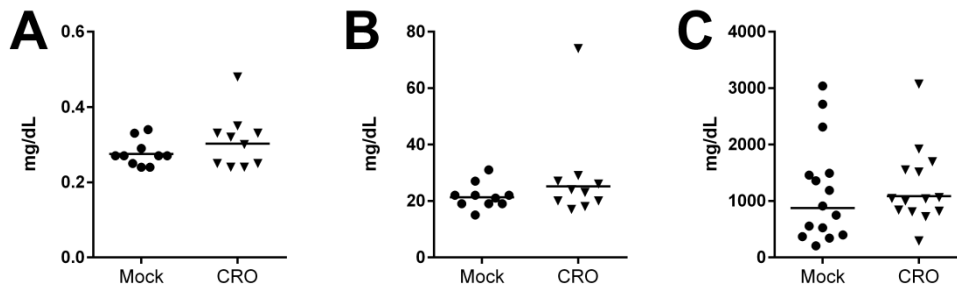
PBS-infected, ceftriaxone-treated; UPEC-infected, PBS-treated; and UPEC-infected, ceftriaxone-treated mice (**A**). Gomori trichrome staining of renal cortex illustrates healthy kidney histology in mock-infected mice which were treated with ceftriaxone (**B**). UPEC-infected mice that had resolved pyelonephritis via ceftriaxone-treatment demonstrated minor glomerular sclerosis, but displayed otherwise normal kidney architecture (**C**).



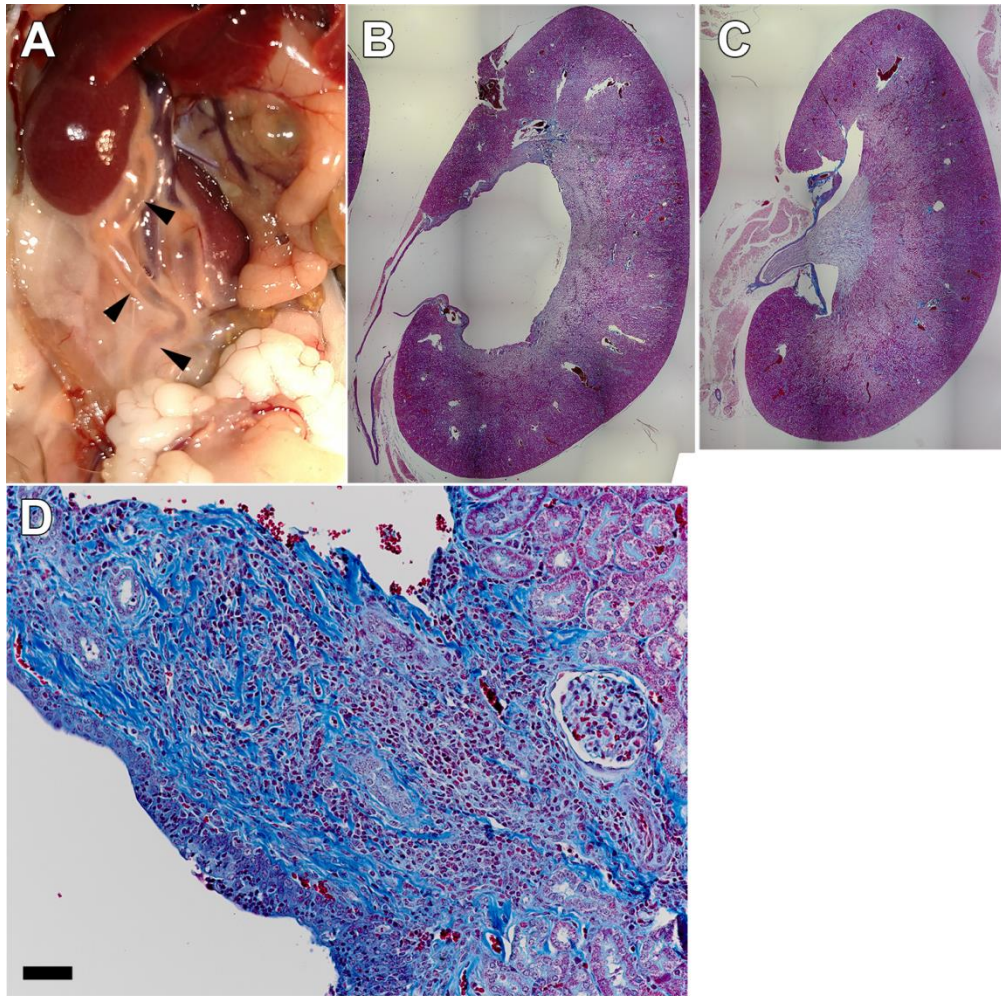


**Figure 5. Pyelonephritic scars demonstrate progressive inflammation.** Gomori trichrome staining of UPEC-infected mice 28 days post treatment demonstrated collagen deposition in place of cortical tissue (**A**, **B**). This was accompanied by dramatic capsular thickening over the scar (black arrowheads) and a cellular infiltrate (**C**). The inflammatory infiltrate appeared to be mostly lymphocytic (**D**).





**Figure 6. Ceftriaxone-treated mice do not develop markers of end-stage renal disease at 5 months post-treatment.** PBS-infected mice (Mock) and UPEC-infected mice (CRO) were treated with ceftriaxone 5 dpi and aged to 30 weeks. Serum was analyzed for creatinine (**A**) and blood urea nitrogen (**B**). Most UPEC-infected mice demonstrated normal creatinine and BUN, but one mouse that displayed bilateral scars demonstrated remarkably high creatinine and BUN. Urine protein was also quantified (**C**).



**Figure 7. Infection resolution leaves residual hydronephrosis 5 months post-treatment.**

Male C3H/HeN were treated with ceftriaxone 5 dpi and aged to 30 weeks to examine the development of chronic kidney disease. The majority UPEC-infected, ceftriaxone-treated males developed gross hydronephrosis with dilated ureters (**A**, black arrowheads). Histopathology of Gomori trichrome stained sections confirmed hydronephrosis in UPEC-infected, ceftriaxone-treated mice (**B**), while PBS-infected, ceftriaxone-treated mice displayed normal kidney architecture without noticeable hydronephrosis (**C**). UPEC-infected animals also displayed evidence of fibrosis with active inflammation, despite resolution of infection (**D**).

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## CHAPTER FIVE

### CONCLUDING REMARKS AND FUTURE DIRECTIONS

Urinary tract infections (UTI) represent one of the most sex-discrepant infectious diseases in humans, with wide disparity in sex-specific incidence of infection throughout the lifespan (1-3). However, technical barriers in preclinical modeling have hampered progress in defining host and microbial mechanisms responsible for the sex differences observed in UTI. This body of work developed a novel murine model to interrogate sex discrepancies in UTI pathogenesis, concretely defined a previously unrecognized hormonal influence on infection, and furthered our understanding of bacterial and host mechanisms in pyelonephritis.

Unlike in female mice, routine transurethral catheterization of the bladder in male mice is technically prohibitive. Previous murine models have utilized catheterization to instill uropathogens into the urethra of male mice (4, 5). These models infect the murine prostate, but fail to reproducibly infect the bladders and kidneys. Consequently, the lack of a tractable male model of UTI has precluded a detailed examination of host and microbial mechanisms underlying male urogenital infections and the sex discrepancies observed in UTI. In response to this deficit, we developed a minimally invasive surgical technique that bypasses simple anatomic differences between sexes to permit infection of the murine urinary tract in both male and female hosts.

Using this model, we revealed the first evidence of an intracellular bacterial community pathway utilized by uropathogenic *Escherichia coli* (UPEC) in male hosts. Compared with females, males of two mouse strains displayed a striking predilection for severe and/or chronic UTI. Further, C3H/HeN males developed more severe pyelonephritis, as well as 100% penetrant

renal abscess (a complication that is rare in female mice). Within the abscess, UPEC formed a dense nidus of intratubular, biofilm-like communities that were sheltered from surrounding inflammatory cells by a fibrotic capsule likely built on tubular remnants. Male susceptibility to severe UTI was mediated by testosterone exposure, and androgenized females likewise developed more robust pyelonephritis and chronic cystitis. Testosterone or its derivatives stimulated this phenotype via activation of the androgen receptor in both sexes.

We further used the male model as a platform for modeling severe pyelonephritis, addressing questions about ascending abscess pathogenesis and the sequelae of severe infection following antibiotic treatment. Of note, our mini-surgical model also consistently yields infection of the prostate in males. Although not detailed here, we found preliminary evidence from gentamicin protection assays of prostate tissue to suggest the existence of intracellular invasion and growth by UPEC during murine prostatitis; additional microscopy studies are required to definitively localize whether an intracellular niche exists. Collectively, these studies demonstrate the utility of the male surgical model outside of sex-comparison research.

### **Androgen receptor influence on UTI susceptibility**

The finding that androgen receptor (AR) activation augments UTI susceptibility opens up vital avenues for further exploration. Primarily, this lends powerful genetic tools to scrutinize the cell and/or tissue types responsible for androgen-mediated susceptibility to UTI. While our data from lipopolysaccharide-insensitive C3H/HeJ mice suggests that mechanisms outside of innate inflammation may be responsible for our phenotype, we can nonetheless pursue reciprocal bone marrow transfers with Tfm mice to determine the relative contribution of the hematopoietic versus epithelial/stromal compartment to androgen-mediated UTI susceptibility. Further, strains

of C57BL/6 mice with floxed alleles of the AR are available to further dissect the specific inflammatory cell lineages or bladder and/or kidney cell types responsible for our phenotype. We thus propose to begin these experiments in the available C57BL/6 background while creating analogous tools in C3H males because of the much more robust sex-discrepant and pyelonephritis phenotypes.

This exploration parallels our quest to identify specific genetic pathways which contribute to androgen sensitization. The exact mechanism which androgens elicit to provoke UTI susceptibility is not clear. In an unbiased approach, we have initiated RNA-seq experiments comparing the renal transcriptomes of acutely-infected wild-type males, wild-type females, castrated males, re-androgenized castrated males, and androgenized females. While androgenized hosts develop chronic cystitis more frequently than nonandrogenized hosts, it is possible that this difference may be due to continual reseeding of the bladder from the infected kidneys. Supporting this hypothesis, acute cystitis proceeds identically in male and female mice of multiple mouse strains, while males consistently develop more robust pyelonephritis. Thus, we postulate that androgen-induced susceptibility in the kidney is the primary driver of chronic UTI in male hosts.

We have found preliminary data illustrating that androgenized hosts display remarkable increases in the oxidative stress of the kidney prior to and during infection. Treatment with the antioxidant, N-acetylcysteine, attenuated acute and chronic pyelonephritis in males, suggesting that androgen-mediated increases in oxidative stress within the kidney may be a contributor to UTI susceptibility. Genes that are known to control oxidation in the kidney, such as NADPH oxidase, can be regulated by testosterone (6). Further exploration of this hypothesis by quantifying expression of reactive oxygen species-producing enzymes in androgenized kidneys,

testing UTI susceptibility in mice genetically deficient in these enzymes, and exploring additional antioxidant therapeutics in male mice is warranted.

Open questions remain on the hormonal influences on UTI. While we find it unlikely that perturbations in other hypothalamic-pituitary-gonadal axis hormones (including GnRH, FSH, and LH) influence UTI susceptibility, these could be more thoroughly tested in turn. Our data clearly suggest that estrogen depletion does not alter chronic outcome in female hosts, but the effects of estradiol on acute pathogenesis in females remain incompletely defined. Indeed, there are conflicting data showing increased UTI susceptibility from both estrogen depletion and elevation in murine hosts, depending on the model used (7-9). Some of this may be explained in part by concurrent fluctuations in testosterone, a previously unappreciated influence on UTI.

At a glance, our findings may appear to represent a stark contradiction – males develop more severe UTI than females when infected via our surgical procedure, but nonandrogenized humans (girls and women) account for the vast majority of UTI cases (2). In fact, the finding that testosterone treatment in female mice perturbs UTI susceptibility extends this paradigm to female populations as well. As detailed in previous chapters, distinct human populations support our hypothesis. Notably, women with polycystic ovarian syndrome (PCOS), a hyperandrogenic state, demonstrate increased incidence of UTI (10-12), and in preclinical models of PCOS, mice overexpressing LH develop spontaneous pyelonephritis (13-16). The development of ascending infection was attributed almost entirely to elevated estrogen in these models, but our data suggest that increased testosterone may in fact be the culprit. These models have not been specifically tested for UTI susceptibility with ascending infection models, and doing so would strengthen the causality between hyperandrogenemia in PCOS and increased UTI incidence. The influence of other hormonal perturbations in this model on UTI susceptibility could be ruled out via

pharmacologic antagonism of specific signaling pathways (*i.e.*, tamoxifen blockade of the estrogen receptor).

Our hypothesis that the postnatal surge in testosterone in male infants increases UTI susceptibility in this population has been detailed in previous chapters. To support this hypothesis, we are collecting additional serum and urine samples from male infants less than 6 months of age with and without UTI to quantify testosterone levels. Female infants also display a surge in hypothalamic-pituitary-gonadal axis hormones during the first 6 months of life with a minor increase in circulating testosterone, albeit still dramatically less than the pubertal levels seen in male infants (17). This burst of androgens may explain, in part, the increased risk of UTI in male infants. Further, we have shown that antagonism of the AR with flutamide results in modest attenuation of UTI in androgenized hosts. Androgen deprivation therapy with newer, more effective nonsteroidal anti-androgens, such as bicalutamide or enzalutamide, could represent a fruitful therapeutic avenue in certain cases of recalcitrant UTI. However, the efficacy of these improved AR antagonists remains to be tested in murine models.

### **UPEC intratubular abscess communities**

The discovery of UPEC intratubular communities within developing renal abscesses furthers our understanding of bacterial pathogenesis during pyelonephritis. UPEC rapidly replicate in the tubular lumina to form an intra-nephron nidus at the center of the nascent abscess. Notably, these populations of bacteria are sheltered from phagocytic attack by the development of a fibrotic capsule and the related inability of neutrophils to cross the tubular epithelia. Based on the fact that C3H/HeJ succumb to disease between 3 and 7 dpi, a time frame when immunocompetent mice develop renal abscess, we posit that this fibrosis surrounding the abscess

communities protects the host from severe sepsis, albeit while also providing a privileged niche for UPEC growth and persistence in the kidney. This fibrosis (as well as tubular occlusion) may also limit antibiotic diffusion to the bacterial nidus during treatment. These bacteria already appear to adopt a biofilm state, and it is well accepted that poor circulation and hypoxia present in abscesses may limit antimicrobial efficacy (18). This combination of factors may underlie the need for prolonged antibiotic treatment in pyelonephritis (19).

However, the details of the development and composition of the fibrotic capsule are unknown. Previous reports have found that microinjection of UPEC into the tubular lumen results in the development of mini-abscess, where bacteria are unable to cross the basal lamina. Further, Melican and colleagues present some evidence that clotting may limit bacterial dissemination from the colonized tubule (20). We suspect that the peritubular capsule is at least partially composed of type IV collagen, plus coagulation components such as fibrin and thrombin. Further investigation of the capsular components and pathways responsible for this process could prove to be of therapeutic interest. We also observe death of tubular epithelial cells surrounding the developing abscess community. The role of this cell death, as well as the activity of peritubular myofibroblasts, in fibrosis development or abscess community formation remains to be further explored.

During the development of the abscess communities, presumably before the development of fibrosis, we still see the inability of inflammatory cells to access the growing UPEC microcolonies within the tubular lumen. Thus, bacterial mechanisms may protect or prevent neutrophil transmigration across the renal epithelia at these early time points. Known mutants in UPEC immunomodulatory virulence factors, such as YbcL (21, 22), could be tested in C3H/HeN males to examine this hypothesis. In this sense, the UPEC abscess community may be analogous

to the IBC, serving as a protected niche for UPEC while nearby inflammatory cells, which have correctly located the bacterial colonies, cannot reach the biofilm community to phagocytose bacteria (23). Indeed, similar bacterial mechanisms may facilitate this subversion of the innate response in both pathways.

In the same vein, a single invasion event and robust expansion gives rise to the development of a clonal IBC (24). We find that abscess communities were focal, interspersed between non-colonized, healthy areas of renal parenchyma. Further, the diseased area of the abscess follows a papillary duct unit subtended by associated nephrons. Based on this, we speculate that a small, founder population of UPEC gains access to the lower reaches of a collecting duct. Bacteria then undergo robust clonal expansion to colonize multiple associated nephrons. This is further supported by the precipitous rise of UPEC communities in the renal parenchyma between 5 and 6 dpi, while we see no evidence of bacteria in the tubules prior. We are currently exploring if these bacterial populations are of clonal origin, and whether bottlenecks shape the UPEC community during its establishment.

We have invested some time in searching for UPEC virulence factors which may specifically increase pathogenicity in the male urogenital tract. We found that type 1 pili are essential for abscess development in male C3H/HeN mice, demonstrating a previously unrecognized role for type 1 pili in virulence within the kidney. This area also provides fertile ground for future research, as type 1 pili may direct UPEC-epithelial or interbacterial interactions (25, 26). In addition to testing other chaperone-usher pili encoded in UPEC genomes (27, 28), promising targets include genes necessary for biofilm formation, such as those encoding curli or cellulose production (28, 29). Utilizing androgenized females in conjunction with males would help elucidate if the putative virulence factors were specifically altering infectivity in male-specific



anatomic niches (*e.g.*, the prostate or epididymis) or were necessary for severe pyelonephritis and abscess development. Unbiased approaches, such as laser capture microdissection (30) of the UPEC abscess community or other *in vivo* bacterial expression profiling, could aid in developing additional targets.

It is notable that the urosepsis UPEC isolate CFT073 likewise causes ascending renal abscesses in androgenized hosts. However, we observed that males began to succumb to CFT073 infection at 2 wpi, presumably from sepsis, while we rarely saw death in UTI89-infected males. In females, CFT073 is a far less fit bladder pathogen than UTI89, exhibiting attenuation at both acute and chronic time points in the bladder and similar bacterial burdens in the kidney (31). This suggests that CFT073 possesses an advantage in survival or propagation in the androgenized kidney, or encodes tools that allow dissemination out of the abscess community or colonized tubule. We have not yet detailed the histopathology of CFT073 abscess communities in the kidney. The male surgical model could also be used to interrogate CFT073 virulence factors necessary for septic dissemination following renal ascension.

### **Pyelonephritic scarring**

The modeling of the long-term sequelae of pyelonephritis has been limited by the inadequate ability of current preclinical models to develop severe disease. We thus exploited our male surgical technique to develop a ceftriaxone-treatment model of severe pyelonephritis. We found that ceftriaxone-treated males develop renal scars and hydronephrosis following resolution of infection. We are currently exploring if these events may compromise kidney function at distant time points following pyelonephritis, and preliminary data indicate this to be true. Most importantly, this model unveils almost limitless new directions to investigate post-pyelonephritic

fibrosis, the development of hypertension and chronic kidney disease following upper-tract UTI, and a potential causative relationship between UTI and hydronephrosis.

Most notable in our observations of the pyelonephritic scars was the accompaniment of ongoing inflammation within and near regions of scar at 1 and 5 months post-resolution. This finding suggests that a progressive process may contribute to the development of renal disease rather than the cumulative loss of renal cortex from infection insult and consequential fibrosis. Ongoing work will illuminate the specific cellular infiltrates present in these scars, their temporal development beginning with active infection, local and systemic inflammatory mediators being secreted, and the respective contributions of these infiltrates and mediators to scar development and progressive renal disease (*i.e.*, through depletion). These lymphoid aggregates present within the scar demonstrated a resemblance to the previously described submucosal lymphoid aggregates described in the bladder of mice and humans developing chronic UTI (32-34), and similar processes may facilitate the recruitment of this ectopic lymphoid tissue during and following UPEC infection regardless of the infected site. Links between UPEC-induced inflammation and the inciting of host fibrosis pathways are currently being explored.

UPEC-infected male C3H/HeN treated with ceftriaxone and aged to 30 weeks surprisingly developed nearly 100% penetrant hydronephrosis. This suggests that there is active remodeling in the bladder, kidney, or both following recovery from UTI which gives rise to the development of hydronephrosis. Other studies have not observed this phenotype in female mice that resolved pyelonephritis, suggesting that hydronephrosis development may depend upon severe pyelonephritis, renal abscess, or androgen effect. The cause of this phenotype and its potential influence on future UTI susceptibility is an area that requires further investigation.

We did not find convincing evidence of end-stage renal disease (ESRD) in UPEC-infected, ceftriaxone-treated animals except in one mouse that developed severe, bilateral scarring. Given that gross scarring develops in only a minority of animals, a greater infectious scarring insult may be required to develop ESRD in this model. While we find it less likely, it may be that pyelonephritic scarring does not lead to progressive ESRD, but rather just the functional loss of cortical tissue to fibrosis which, when combined with unrelated renal insults later in life, sums to potentiate ESRD. We plan to investigate other more common potential sequelae of scarring and resolved pyelonephritis, such as hypertension. Further research investigating the mechanism of pyelonephritic fibrosis and chronic kidney disease promises to help guide the development of therapeutic modalities that block the fibrotic pathways or processes contributing to the progression of this important disease.

### **Concluding remarks**

In this work, we have developed a novel model to study sex differences in UTI pathogenesis. We specified male-specific attributes driving more robust pyelonephritis and concurrently filled a void in the field for modeling severe renal infection. In ascending renal abscesses, UPEC organized into intratubular, biofilm communities which were protected from surrounding inflammatory cells by peritubular fibrosis. The development of chronic UTI was dependent on testosterone, and androgenization enhanced susceptibility to severe UTI in female mice, signaling specifically through the androgen receptor. These findings give mechanistic insight into why certain male and female patient populations have increased UTI susceptibility. While this body of work significantly advances our knowledge of UTI pathogenesis, it also opens countless new avenues for future exploration.

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## APPENDIX ONE

### *ESCHERICHIA COLI* IN URINARY TRACT INFECTIONS

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#### **Abstract**

Urinary tract infections (UTIs), caused primarily by uropathogenic *Escherichia coli* (UPEC), are among the most common bacterial infections in humans, costing billions of dollars annually in medical expenses and causing significant morbidity with recurrent and chronic disease. Many details of the molecular pathogenesis of UPEC UTI have been unveiled through recent advances in a murine model of cystitis. Following ascension to the bladder, UPEC bind and invade superficial facet cells of the uroepithelium via type 1 pili. Uropathogens are able to evade innate cellular defenses, in part, by proceeding through an intracellular cascade in which UPEC replicate within the facet cell cytoplasm, forming densely packed intracellular bacterial communities (IBCs). Egress of filamentous bacteria from IBCs can initiate subsequent rounds of IBC formation, thereby perpetuating acute infection. UPEC also invade the transitional



epithelium, establishing latent, non-replicating reservoirs that resist antibiotic therapy and may initiate same-strain recurrent infection. UPEC employ a variety of additional molecular strategies to facilitate infection and to delay and subvert the host immune response. Finally, novel therapeutic and preventive strategies such as anti-virulence compounds and vaccines may be necessary to combat the growing threat of antimicrobial resistance.

## **Introduction**

The human urinary tract is one of the most common sites for bacterial infection. Uncomplicated urinary tract infections (UTIs) account for 8 to 14 million medical visits annually and almost \$4 billion in medical expenditures each year in the United States (1, 2). By the fourth decade of life, half of all women report having had at least one UTI. Of those, 25% will experience a second UTI and 3% will experience a third recurrence within six months after treatment of the initial infection (3, 4). Molecular typing, including that by restriction fragment length polymorphism analysis, suggests that up to two thirds of recurrent infections arise from the same bacterial strain as that causing the initial infection (5). Same-strain recurrent infections have been observed up to 3 years after an initial UTI (3, 6). As these chronic and recurrent UTIs cause a substantial deterioration in patients' quality of life (4, 7), a more thorough understanding of the processes underlying the initiation and recurrence of these infections is needed.

Uropathogenic *Escherichia coli* (UPEC) cause up to 90% of all culture-confirmed UTIs (3) and represent the leading cause of both community-acquired and healthcare-associated UTIs. Other bacteria, including *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus* spp., also cause community-acquired infections, while enterococci, *Pseudomonas* and *Candida* are prominent among healthcare-associated infections. In most

cases, UTI appears to develop along an ascending route, with urethral ascent following periurethral colonization by the offending organism. These ascending uropathogens then colonize the bladder epithelium using specific sets of virulence factors. Infection may be limited to the bladder (cystitis) or ascend to the upper urinary tract causing pyelonephritis. Just as multiple *E. coli* pathotypes (*e.g.*, EHEC, ETEC, EAEC) cause distinct gastrointestinal diseases (8), UPEC strains wield distinct repertoires of virulence determinants to cause disease and persist in these separate niches within the urinary tract.

In order to successfully colonize the urinary tract, UPEC must circumvent formidable host defense systems, which are mechanical, biochemical and immunologic in nature. The traditional view holds that bacterial cystitis is considered an extracellular, self-limiting acute infection. However, much of our current knowledge of the molecular intricacies of cystitis has been more recently revealed using a robust murine model of ascending UTI. The pathogen evades host defenses, in part, by proceeding through a well-documented intracellular cascade where UPEC (and other uropathogens such as *Klebsiella*) adhere to and invade superficial epithelial cells of the mammalian bladder (9-23). UPEC then rapidly replicate inside these host cells, initially forming loose collections that subsequently coalesce into densely packed intracellular bacterial communities (IBCs) (9). Murine and *in vitro* data have been corroborated by detection of IBCs in human samples (24), suggesting that the IBC pathway observed in the murine model recapitulates human cystitis.

In the following sections, though it is not feasible to exhaustively review decades of work in UTI pathogenesis, we will detail some of the more recently revealed molecular attributes of pathogen and host that define the conversation between these two entities and determine the outcomes of encounter. Given our personal experience, many of these paradigms will be more

relevant to cystitis than pyelonephritis. In addition, we will cover current clinical problems in UTI and the prospects for novel therapeutics and preventives against these highly prevalent and recurrent infections.

## **Epithelial Adhesion and Pili**

Bacteria that fail to adhere to host tissues are unable to cause disease. This axiom is true of many bacterial pathogens, especially those infecting epithelial surfaces, and has been clearly demonstrated in the development of cystitis and pyelonephritis caused by UPEC, which produce a variety of surface-associated structures that serve as adhesins (25). Most important for UTI, UPEC may produce a variety of heteropolymeric, proteinaceous extracellular fiber-like organelles, termed pili, that mediate adhesion to specific receptors on the uroepithelium. UPEC genomes typically contain multiple discrete loci encoding highly resilient pili that are assembled via the canonical chaperone/usher secretion pathway (25), as detailed elsewhere in this text. The type 1 pilus mediates bacterial adherence to mannosylated uroplakin proteins on the luminal surface of the bladder epithelium (26), while type P pili bind globoseries glycolipids found on kidney epithelial surfaces (27). These fimbriae are critical for successful infection of the urinary tract, with UPEC heavily relying on type 1 pili to colonize and invade the bladder epithelium (9, 18, 28-30).

The regulation of chaperone-usher pili by UPEC is a complex process. Type 1 pilus expression from the *fim* operon is controlled on the genetic level by a process known as phase variation, where bacteria may alternate between piliated (phase-ON) cells and nonpiliated (phase-OFF) states. This is accomplished by inversion of a DNA segment, *fimS*, containing two 9-bp repeats flanking the *fimA* promoter (31, 32). During phase-ON, the orientation of *fimS*

positions the *fimA* promoter toward the *fim* operon, allowing transcription of type 1 pili structural subunits; transcription halts in the inverted phase-OFF position (33). Site-specific inversion of *fimS* is catalyzed primarily by two linked recombinases, FimB and FimE (34), but may also be mediated by other recombinases elsewhere on the chromosome (35). In addition, a complex regulatory network controlling the modulation of expression of type 1 pili due to changing environmental conditions is beginning to be elucidated. Many effectors, such as RpoS and ppGpp, alter type 1 piliation by regulating expression and activity of recombinases (35-38). Many other regulatory proteins also influence *fim* expression, including Lrp, CRP-cAMP, the global regulator H-NS, and integration host factor (IHF) (39-42). Mechanisms may include a proper DNA supercoiling of the switch site, aided by the binding and involvement of Lrp, IHF, and H-NS to the region, consequently affecting the transcriptional promoter and/or the recombination process (42-46). Additionally, the QseC sensor kinase and the transcriptional regulator MarA have been recently identified as regulators of type 1 expression (47-49). These emerging details suggest an intricate complexity of regulatory components that combine to tightly control UPEC expression of this important urovirulence factor.

## **Toxins**

UPEC may secrete a variety of toxins that damage host epithelia and promote pathogenesis within the urinary tract. One of the earliest recognized toxins is the  $\alpha$ -hemolysin, named because it mediates eukaryotic cell lysis at high concentrations, an activity which might promote the release of previously sequestered nutrients that can be used by UPEC during infection. HlyA is distributed across a wide range of bacterial phyla and is a member of the repeats-in-toxin (RTX) family of proteins. UPEC strains that express HlyA are associated with

more extensive tissue damage and disease severity in the urinary tract (50-52). HlyA requires activation of its protoxin form via post-translational acylation of two lysine residues. The activated toxin disrupts eukaryotic cells by oligomerizing at higher toxin concentrations to form pores in the host cell membrane in a calcium-dependent fashion, leading to cell lysis (53, 54). Further, recent studies by Dhakal and Mulvey have shown that at sublytic levels, HlyA prompts the degradation of host proteins (*e.g.*, paxillin) involved in intercellular adhesion and host cytoskeletal integrity (55). Thus, HlyA may be a primary bacterial effector driving the exfoliation of superficial bladder epithelial cells that is observed early in the course of UPEC infection (29). Additionally, as will be discussed further below, HlyA may inhibit proinflammatory cytokine production by bladder epithelial cells (55). These findings demonstrate interesting additions to the growing list of secondary functions of pore-forming toxins and establish a more complete phenotypic basis for the high prevalence of HlyA carriage among UPEC isolates.

Cytotoxic necrotizing factor 1 (CNF1) is expressed by ~ 40% of UPEC isolates and 30% of *E. coli* from gastrointestinal pathotypes (56). Like HlyA, CNF1 may modulate a variety of epithelial and leukocyte functions to propagate infection in the urinary tract. The toxin constitutively activates small Rho GTPases such as Rac1, causing *in vitro* alterations that include rearrangement of the actin cytoskeleton, enhanced uroepithelial membrane ruffling and uptake of bacteria, uroepithelial cell apoptosis, and decreased PMN phagocytic activity (13, 57-61). The implication is that *in vivo*, CNF1 likely promotes invasion of UPEC into the uroepithelium in addition to suppressing phagocytic activity. Interestingly, CNF1 localizes to the bacterial periplasm and appears to be delivered to host cells via the liberation of outer membrane vesicles, which ferry the CNF1 as cargo to a fusion event with susceptible host cell membranes (61). Of

note, HlyA or CNF1 mutants tested in multiple UPEC backgrounds generally remain virulent in animal models of infection (52, 56, 62), suggesting that the mere encoding of these toxins by UPEC is not essential for infection, but that spatiotemporal regulation of toxin expression and secretion contributes to pathogenicity during UTI.

Several additional putative toxins are being uncovered that impact *E. coli* UTI. Mobley and colleagues recently utilized *in vivo* induced antigen technology to identify a putative RTX exoprotein, TosA, that is exclusively expressed *in vivo* during experimental UTI (63). The *tosA* gene was found to be enriched among urinary isolates of *E. coli* compared to fecal isolates, and *tosA* presence predicted successful colonization of the murine urinary tract (64), while deletion of *tosA* led to significant attenuation in the urinary tract (63). Although the precise mechanism remains unclear, TosA may enhance adherence in the kidneys and increase survival of UPEC in invasive niches (65). As discussed below, other bacterial exoproteins, such as YbcL, may further augment modulation of innate immunity when released during UPEC infection (66). Future studies will undoubtedly specify novel UPEC toxins, further delineate their mechanisms of action, and reveal the spatiotemporal regulation strategies that promote pathogenesis within the urinary tract.

### **Other virulence determinants**

A conserved mammalian host strategy during episodes of infection, including UTI, involves exquisitely developed systems that sequester essential nutrients and thereby limit their availability to bacterial pathogens. Notably, UPEC has evolved to reside in two distinct host environments, namely the gastrointestinal and urinary tracts. Consequently, the pathogen's transition from the comparatively nutrient-rich gut involves substantial upregulation of nutrient

acquisition systems to permit colonization of the nutritionally poor urinary tract (67).

Specifically, the acquisition of transition metals represents a constant battleground between a bacterial pathogen and its mammalian host. Iron acquisition by UPEC has emerged as the prototypical model for high-affinity nutrient uptake to combat host-mediated limitation.

UPEC and other extraintestinal pathogenic *E. coli* encode a greater variety of defined and putative metal transporters compared to *E. coli* K-12 and other commensals (68). Further, *E. coli* isolates causing UTI possess multiple functionally redundant systems mediating iron uptake, suggesting that the urinary tract represents an iron-limiting environment during infection (68, 69). Many of these systems rely on bacterial secretion of low molecular weight  $\text{Fe}^{3+}$ -chelating molecules, known as siderophores, that serve to scavenge free and host protein-bound iron. Such iron is then retrieved via receptors that facilitate transport of iron-siderophore complexes into the bacterial cytoplasm. Four distinct siderophore systems have been characterized in UPEC: yersiniabactin, aerobactin, enterobactin, and salmochelin (70). *E. coli* isolates expressing a greater repertoire of siderophore systems may experience a fitness advantage during growth *in vivo* and/or in human urine (71, 72). A recent report also suggests that siderophores may also contribute to virulence by functions distinct from iron scavenging, demonstrating that yersiniabactin can chelate  $\text{Cu}^{2+}$  and consequently increasing resistance to copper stress (73). In addition to siderophores, UPEC employ a marked upregulation of direct heme receptors, including ChuA and Hma, to scavenge free iron during UTI (67, 74).

UPEC has also developed mechanisms to counter the metabolic limitations of the urinary tract. In response to experimental infection, the mouse uroepithelium increases expression of genes involved in glucose import, likely to represent a response to increased cellular energy requirements (67). Consequently, glucose may not represent the primary carbon source for

UPEC *in vivo*, and UPEC may instead utilize short peptides and amino acids as carbon sources during infection (75). Encoding of certain metabolic pathways allows UPEC to avoid stresses introduced by other metabolite-limiting systems evolved by mammalian hosts. For example, activity of the indoleamine 2,3-dioxygenase (IDO) pathway limits tryptophan availability for many human pathogens; because UPEC is able to synthesize endogenous tryptophan, upregulation of IDO during UTI does not limit UPEC growth (76).

As with other encapsulated bacteria, the elaboration of capsular polysaccharides by *E. coli* has been shown to inhibit phagocytosis and increase serum resistance (77, 78). Many UPEC isolates express group II capsules, primarily the K1 polysaccharide, a linear  $\alpha$ 2-8-linked sialic acid homopolymer (50, 79) that is shared by *Neisseria meningitidis* group B. Earlier studies indicated that capsule-deficient UPEC mutants have reduced fitness during experimental UTI, including deficiencies in both the upper and lower urinary tract, though specific deficits in the pathogenic cascade had not been investigated (80-82). More recent data demonstrate that capsule-deficient UPEC mutants are hindered in their ability to aggregate and form IBCs, suggesting that K1 capsular polysaccharide contributes to the coalescence and organization of these IBCs within superficial facet cells, perhaps as a key constituent of the IBC matrix (83). This lack of biofilm-like intracellular aggregation by capsule-deficient mutants may also leave the bacteria more susceptible to neutrophil attack, soluble immune mediators, or antibiotics (83). It is currently unclear whether other capsular serotypes might contribute to IBC formation in a similar fashion and how UPEC capsule specifically promotes virulence within other urinary tract niches.

Additional factors promoting UPEC biofilm properties *in vitro* may also prove to be important *in vivo* during IBC formation. A comprehensive screen of UPEC transposon mutants



identified a variety of novel effectors involve necessary for *in vitro* biofilm growth. Mutations in a subset of these effectors resulted in attenuation during murine UTI, with significantly decreased capacity for IBC formation (84).

### **Invasion of UPEC into the uroepithelium**

The most current paradigm for the progression of cystitis involves invasion of UPEC (or other uropathogens, such as *Klebsiella* species) into superficial bladder epithelial cells (9-23, 85-87). Invasion of UPEC into kidney epithelial cells has also been demonstrated (88, 89), but the ramifications of this event for disease progression are not as clear as in cystitis. In addition to adhesive pili, UPEC invasion into superficial bladder epithelial cells involves numerous host factors (**Table 1**). In many cases, these invasion events result in initial confinement of internalized UPEC within a membrane-bound compartment. Fusiform vesicles, small “chunks” of uroplakin-expressing apical epithelium, normally cycle into and out of the apical membrane to regulate bladder surface area during the accumulation of urine (11). Pharmacological interference with these host processes has been shown to significantly affect numbers of intracellular UPEC (**Table 1**), suggesting that the invasion process, at its core, may be a reflection of UPEC co-opting this normal membrane cycling (discussed later). The use of such agents may therefore represent a novel therapeutic approach to reduce the intracellular bacterial burden as a means to prevent or attenuate UTI.

To date, no bacterial effectors that modulate the host specifically to enhance UPEC invasion of epithelial cells have been identified. The FimH adhesin of the type 1 pilus is both necessary and sufficient for internalization (17, 23, 90). Beyond the membrane-bound intracellular UPEC that may recycle to the surface, a small percentage of internalized UPEC gain

access to the cytoplasm. It is unclear whether this cytoplasmic niche is attained by previously membrane-bound organisms (through a yet-undefined mechanism), or if these cytoplasmic bacteria have followed an alternative cell-entry pathway. Growth of UPEC within the cytoplasm of cultured bladder epithelial cells occurs when detergents are added post-invasion (14), suggesting that escape from the membrane-bound compartment is sufficient to facilitate intracellular replication. This cytoplasmically located subpopulation of UPEC represents the ultimate source for ongoing colonization, robust bacterial expansion within IBCs, and eventual bacterial persistence within the uroepithelium.

### **Intracellular lifestyle of UPEC during cystitis**

Time-lapse fluorescence microscopy of live, explanted infected murine bladders revealed that UPEC proceed through a complex developmental and differentiation pathway during experimental cystitis, multiplying in number and evading host immune effectors by establishing a niche within superficial bladder epithelial cells (91). The developmental pathway includes two independent branches that are distinguished by different patterns of morphological differentiation. At the earliest stage, all of the bacteria exist in a standard bacillary shape (ca.  $1\ \mu\text{m} \times 3\ \mu\text{m}$ ). These early-stage UPEC double every 30 minutes within loosely associated IBCs (**Figure 1**). The majority of the bacteria within an IBC mature through the *first differentiation branch* and synchronously divide with a shorter cell length to yield daughter cells that appear more coccoid (ca.  $1\ \mu\text{m} \times 1\ \mu\text{m}$ ). This coccoid shape allows for more efficient utilization of the intracellular space than the bacillary shape, by approximately 2 orders of magnitude. It has been estimated that  $\sim 10^4$ - $10^5$  organisms occupy the cytoplasm of the infected superficial facet cell, or **pod** (9). As a consequence of the coccoid shape, the architecture of the UPEC community

becomes more spherical (91, 92) (**Figure 1**). Physiological changes accompany these morphological changes, as evidenced by a reduction in growth rate (increase in doubling time to ~45 minutes). As the size of the IBC approaches the capacity of the pod, the coccoid-shaped bacteria at the periphery of the IBC regain a bacillary shape and become highly motile (**Figure 1**). This stage is typically associated with apoptosis and cell death (29, 93-95), providing disruptions in the cell membrane to permit escape of motile UPEC (**Figure 1**) that subsequently attach to naïve epithelial cells or leave the host through micturition.

Meanwhile, a minority of the early-stage intracellular population proceeds through a *second differentiation branch* that results in the formation of non-septate filamentous bacteria (up to 70 µm in length), associated with expression of the cell division inhibitor Sula (91, 96). These filamentous morphotypes egress along with bacillary-shaped bacteria and attach to the luminal surface of the bladder epithelium. Cell division is restored once these filamentous UPEC are extracellular, and they ultimately produce daughter cells of normal bacillary length that remain adherent to the bladder surface (91). The advantages of this second morphotype during fluxing include increased resistance of the filamentous bacteria to neutrophil and macrophage phagocytosis (96, 97). The observation that each intracellular community arises from a single progenitor cell (98) suggests that the signals directing these dual, parallel developmental programs are derived from the host microenvironment.

Following egress, attachment to naïve superficial epithelial cells can initiate subsequent rounds of IBC formation that perpetuate the infection. During experimental UTI in the murine model, the first round of IBC formation proceeds with remarkable synchrony. With typical inocula of UPEC during experimental murine cystitis ( $\sim 10^7$  CFU), several hundred IBCs develop during each cycle. Because human UTI arises from a presumably lower inoculum, cystitis could

be initiated by a single bacterium that gains access to the bladder epithelial cell cytoplasm. With each round of IBC formation, there is significant discharge of bacteria into the urine (bacteriuria) through egress, but the intracellular amplification of UPEC leads to the propagation of infection.

Apoptosis of superficial epithelial cells as a consequence of infection results in the release of cellular debris, including harbored IBCs (24), into the urine. Later during infection, this exfoliation eventually involves most or all of the superficial epithelial layer, exposing the underlying transitional epithelium. Unlike other epithelial layers of the body that completely turn over within days, normal bladder epithelium may require 6 months for turnover of the superficial bladder epithelial cells (99). Exfoliation that follows UPEC infection in mice is accompanied by dramatically activated differentiation machinery, so that a new superficial layer is regenerated within days (29, 100). Although one would imagine that exfoliation of the superficial facet cells (particularly those containing IBCs) might attenuate the acute infection, it conversely sets the stage for the establishment of a quiescent intracellular reservoir (QIR) that has characteristics of a latent infection. In contrast to the invasion of superficial facet cells, where a small proportion of invaded bacteria become free within the cytoplasm, invasion into the transitional cells results in bacteria within a membrane-bound compartment (101) that does not support growth (91). The QIR remains intact following differentiation of the transitional cells into new superficial facet cells. Following experimental cystitis in mice, the QIRs are observed for months (85), are resistant to systemic antibiotic therapy, and can serve as the source for recurrent UTIs (102). Although exfoliated pods and filamentous bacteria are readily identifiable in the urine during UTI in humans, direct evidence of QIR formation in humans is lacking. The use of fluorescent 16s RNA probes was unsuccessful in the identification of QIR within biopsies of children with recurrent UTI (103). This study highlights the need for new approaches to

identify QIRs in human samples, given the suspected few numbers of QIRs and the apparently low metabolic and growth activity of these chronically resident bacteria.

The relevance of the IBC cascade, defined in the murine model, to human cystitis has been validated through multiple independent lines of investigation. The first approach involved the analysis of five different murine host strains (C57BL/6, CBA/J, FVB/NJ, C3H/HeN, and C3H/HeJ) and four clinical isolates of UPEC (from distinct patients with the clinical syndromes of acute cystitis, recurrent cystitis, pyelonephritis, and asymptomatic bacteriuria); the observation that multiple human UPEC isolates and multiple murine genetic backgrounds supported IBC formation indicates that this developmental pathway is not limited to a single UPEC/host pair (104). A second and more convincing approach involved the detection of exfoliated epithelial cells bearing IBCs in urine specimens from women seeking treatment for UTI (24). These authors also observed filamentous bacteria in 41% of these urine samples, demonstrating that bacterial filamentation occurs commonly during human cystitis. Third, in addition to *E. coli* UTIs, filamentous bacteria were observed in urine samples from women infected with *Enterobacter aerogenes*, *Klebsiella pneumoniae*, or *Proteus mirabilis* (24), indicating that the second branch of the developmental pathway is common to multiple Gram-negative bacteria causing disease in the urinary tract. Finally, upon infection with pools of genetically “bar-coded” isogenic UPEC strains, a significant decrease in the diversity of the UPEC population was observed in temporal association with the second round of IBC formation (98). The intracellular paradigm predicts such “bottlenecks” in the diversity of organisms colonizing the bladder (due to the relatively few numbers of epithelial cells that house developing IBCs; **Figure 1**), while exclusive luminal infection would not be characterized by these bottlenecks. Taken together, these data indicate that the paradigm arising from UPEC

infections of mice can likely be translated to the human condition and to infections caused by other uropathogenic bacteria.

### **Chronic cystitis**

The introduction of bacteria into the urinary tract of susceptible murine host strains can also result in a long-term chronic infection characterized by ongoing high bacterial loads and inflammation, including robust neutrophil infiltration (105). This phenotype appears to arise as the result of an early immune checkpoint involving multiple cytokines (e.g. IL-5, IL-6, granulocyte colony-stimulating factor, keratinocyte-derived cytokine) (105). Of interest, development of this chronic active cystitis could be prevented by the prior treatment with the anti-inflammatory agent, dexamethasone (105). This model provides a tool to elucidate the natural history of UTI and mechanisms that might underlie chronic UTIs.

### **Immune response and evasion**

Innate immunity, particularly those responses initiated by Toll-like receptor 4 (TLR4) recognition of the lipopolysaccharide (LPS) of Gram-negative bacteria, has attracted the vast majority of attention paid to the immune defenses of the mammalian bladder. Early observations that the murine C3H/HeJ background was more susceptible to UTI (as measured by recoverable colony-forming units from bladder and kidneys) were followed by identification of a mutation in the *Tlr4* gene (106, 107). Stimulation of TLR4 activates the classic NF- $\kappa$ B pathway via the adaptor MyD88, IL receptor-associated kinase (IRAK), and other kinases in the host epithelia and immune effector cells, leading to expression of IL-6 and IL-8, which are measurable in the urine of mice and humans with UTI (108, 109). However, a second pathway activated by TLR4

in bladder epithelia also results in IL-6 and IL-8 expression (110). In this model, LPS stimulation of TLR4 triggers an influx of  $\text{Ca}^{2+}$  into the intracellular space, followed by an increase in cyclic AMP (cAMP) levels. The cAMP response element binding (CREB) protein then becomes phosphorylated, resulting in additive promotion of the transcription of both cytokines. Data from selective blockade of these signaling cascades suggests that epithelial secretion of IL-6 may be even faster via cAMP/CREB than through the canonical TLR pathway (110).

On the basis of these and other observations, and given that IL-8 is a recognized chemoattractant for neutrophils, the defect in UTI resistance seen in C3H/HeJ (i.e., functionally TLR4-deficient) mice has been attributed primarily to a failure to recruit neutrophils to the bladder upon UPEC infection (111). However, recent data suggest that TLR4 may mediate host defense mechanisms beyond the recruitment of immune cells. Epithelial invasion studies in C3H/HeJ mice revealed a 10-fold increase in intracellular bacteria 1 h post infection, compared with C3H/HeN (wild-type TLR4) mice (112). This phenotype was found to relate to two additional TLR4-stimulated processes. First, intracellular cAMP inhibited Rac-1, a host protein necessary for cytoskeletal rearrangements that accompany bacterial invasion, thus potentially rendering superficial bladder epithelial cells less receptive to bacterial internalization. Second, bladder epithelial cells can actively expel nascently invaded bacteria in association with the normal exocytosis of fusiform vesicles that accompanies bladder distension. Expulsion via this membrane-cycling mechanism is dependent on TLR4, cAMP, Rab27b, and caveolin-1 (11, 85, 112, 113).

Bacterial products beyond LPS may also stimulate TLR4 and other TLRs expressed on the uroepithelium and/or resident immune effector cells of the urinary tract. Both type 1 and P

fimbriae appear to stimulate TLR4, via FimH and a ceramide linkage of globoside receptors, respectively (114-117). TLR5 stimulation, responding to bacterial flagellin, also contributes to acute inflammation, as mice lacking TLR5 experience more robust infections of the bladder and kidneys (118). Human ureter epithelial cells grown *in vitro* showed a response to flagellin but not to LPS, suggesting that TLR5 may be important during the initial inflammatory response (119). Finally, mice lacking TLR11 (associated with a pseudogene in humans) were shown to be more prone to upper tract UTI (120).

Neutrophils play a critical role in controlling UPEC UTI; peripheral neutrophil depletion of C3H/HeN mice resulted in a drastic impairment in clearance of UTI, with higher bladder and kidney bacterial loads than in the TLR4-deficient C3H/HeJ background (where PMN response is diminished and delayed) (121). This suggests that other mediators also shape the timing and extent of the PMN response to infection in the mammalian bladder. UPEC infection was demonstrated to induce the secretion of granulocyte colony-stimulating factor (gCSF) in the bladder, and antibody-mediated depletion of this cytokine reduced PMN influx following UPEC infection (122). Similarly, plasminogen activator inhibitor type 1 (PAI-1) influences cell migration through its effects on integrin binding; upon UPEC infection of mice lacking PAI-1, kidneys bore significantly higher bacterial burdens and fewer PMN infiltrates than wild-type counterparts did (123). The secretion of a number of soluble antibacterial compounds into the urinary tract is also induced during infection. The human cathelicidin LL-37, a short antibacterial peptide, is detectable in the urine during human cystitis, and mice deficient in its ortholog (CRAMP) demonstrate increased susceptibility to pyelonephritis (124), though its impact on cystitis remains incompletely explored. Multiple members of the defensin class of antimicrobial peptides are also produced locally during UTI, and the participation of these



molecules in the host-pathogen conversation remains a fertile area for study. Of note, UPEC also elicit the production of nitric oxide via induction of the iNOS gene (100), although the impact of this on infection outcome is questionable, as mice deficient in iNOS have not shown increased susceptibility to UTI (125, 126).

Despite this substantial provocation of the innate immune system during infection, UPEC is able to persist in the urinary tract, withstanding mechanical defenses, acute immune insults and chronic immune surveillance. Early in infection UPEC must survive a highly inflammatory soluble and cellular response, specifically a robust influx of neutrophils mediated by local secretion of multiple cytokines and chemoattractants. A growing body of data demonstrates that UPEC employs a variety of strategies to delay, dampen, attenuate, and subvert these acute host responses to infection.

As detailed above, multiple steps in the pathogenic cascade of cystitis (*e.g.*, epithelial invasion, IBC formation, and bacterial filamentation) illuminate critical UPEC strategies for immune evasion. Of primary importance, the intracellular niche provides a haven for bacterial multiplication, subverting PMN phagocytic activity. By video microscopy, PMNs can be observed readily engulfing luminal bacilli within hours of murine infection. Meanwhile, UPEC that have successfully invaded (and are replicating within epithelial cells) are sheltered from this attack; PMNs are clearly able to locate infected superficial epithelial cells but cannot access the expanding bacterial community (127). Mature IBCs also yield a population of organisms that assume a filamentous morphology, subsequently traversing the luminal surface to initiate additional rounds of IBC formation in naïve epithelial cells. Again, during this time of transition, bacillary forms of UPEC are readily engulfed, while the filamentous bacteria resist phagocytosis, even when coming in direct contact with PMNs and macrophages (96, 97). Egress

of bacteria from mature IBCs coincides with an enrichment of filamentous forms within the bladder lumen, consistent with a selection process arises where bacillary forms are preferentially taken up by host phagocytes (127); this preferential engulfment is also supported by *in vitro* flow cytometry studies (97). Confirming that a true conversation is taking place, filamentation was not observed in TLR4-deficient backgrounds and was found to be induced *in vitro* by exposure of bacillary UPEC to LPS-activated macrophages (97, 127).

In a related arena, several recent studies suggest that UPEC, unlike laboratory or commensal strains of *E. coli*, can partially suppress epithelial inflammatory responses. UPEC stabilize I $\kappa$ B, suppressing NF- $\kappa$ B activity in association with increased bladder epithelial cell apoptosis upon exposure to UPEC (128). Further, multiple UPEC isolates elicit lower levels of IL-6 and IL-8 from uroepithelial cells and are able to block secretion of these cytokines upon co-inoculation with known NF- $\kappa$ B stimuli (129, 130). Genes involved in LPS biosynthesis are also important for this phenotype, suggesting that LPS modifications imparting a less stringent inflammatory phenotype may underlie the nonstimulatory properties of UPEC (130, 131). In addition, lack of the OMP chaperone SurA abolishes the immunosuppressive phenotype (130); reduced levels of the SurA-dependent LPS transport protein LptD may alter the presentation of potential nonstimulatory LPS (132).

However, LPS differences alone do not account for cytokine suppression (133), and it is likely that other mechanisms and UPEC effectors also contribute to suppressing cytokine secretion. For example, NF- $\kappa$ B signaling may be inhibited by sublytic concentrations of  $\alpha$ -hemolysin (55, 134). Also, a minority of UPEC, including the pyelonephritis/urosepsis strain CFT073, encode a Toll/IL-1 receptor (TIR) domain-containing protein, termed TcpC, that interacts with the host adaptor MyD88 to inhibit TLR signaling (135). TcpC disruption was

associated with decreased bacterial burden in the kidneys and a reduction in histologically evident renal damage, signifying the importance of this protein during pyelonephritis. Further study of additional signaling pathways that rely on TIR domain-containing proteins (e.g., the TRIF and IL-6/IL-1 signaling cascades) revealed that TcpC also regulated these pathways independent of MyD88, suggesting that TcpC may impact pathogenesis in a broader way (136). Finally, the SisA and SisB proteins, homologues of the immunomodulatory protein ShiA from *Shigella flexneri*, are found in CFT073 and have been recently implicated in immune modulation during UTI (137).

In addition to suppressing proinflammatory cytokine production, UPEC is also able to resist and modulate neutrophil functions (138). UPEC strains are able to resist phagocytic killing in comparison to non-pathogenic *E. coli* isolates, down-regulating expression of PMN genes involved in neutrophil chemotaxis, proinflammatory signaling, adhesion, and migration (138). UPEC isolates also elicit reduced bladder PMN influx *in vivo* and attenuate transepithelial PMN migration in an *in vitro* model (66, 138). UPEC have been shown to induce a number of anti-inflammatory molecules in neutrophils, including indoleamine 2,3-dioxygenase (IDO) (138). IDO induction within PMNs and uroepithelial cells during experimental cystitis resulted in dampened PMN influx, correlated with increased survival of extracellular UPEC (76).

As noted above, UPEC employ the toxins HlyA and CNF1 to neutralize the phagocytic activity of PMNs (60-62, 139-141). A newly identified UPEC effector, termed YbcL, has been shown to inhibit transepithelial migration of human neutrophils via a distinct but as yet incompletely defined mechanism. This effector localizes initially to the bacterial periplasm and is subsequently liberated, suppressing PMN migration in both *in vivo* and *in vitro* models (66). Interestingly, YbcL homologs present in laboratory or commensal *E. coli* isolates are unable to

suppress PMN migration due to a single amino acid mutation, while most UPEC strains encode the suppressive YbcL variant (66). In sum, these anti-neutrophil strategies of UPEC appear important in delaying PMN influx and avoiding phagocytosis at the earliest stages of infection, allowing the pathogen a window of opportunity in which to establish its protected intraepithelial niche.

### **Host genetic susceptibility determinants**

In addition to many bacterial determinants, there are host traits that contribute to disease susceptibility, severity and progression. The strong gender bias in the frequency of UTI has traditionally been attributed to the anatomical differences between sexes (distance from anus to urethral opening, length of the urethra, and permissiveness of the vaginal/perineal microenvironment). Dysfunctional voiding and other urodynamic abnormalities are associated with increased susceptibility to UTI in children (142), and pregnancy also predisposes to UTI. However, it is likely that sex differences and inter-individual differences in susceptibility represent phenotypes that are more complicated than these simple reasons explain. Along these lines, multiple efforts to define host genetic determinants of susceptibility to UTI have been modestly successful. Given the observations outlined in the foregoing section, attention in this realm has been focused on host innate immune genes. However, it has also become clear that susceptibility is both polygenic and environmental, and that genetic determinants of distinct clinical syndromes (asymptomatic bacteriuria, cystitis, and pyelonephritis) must be pursued independently.

### *P blood group.*

Globoseries glycolipids, which serve as receptors for the UPEC P pilus adhesin PapG, also represent P blood group antigens. Early work indicated that individuals expressing the P1 antigen have higher susceptibility to pyelonephritis (143).

### *TLR4.*

As noted above, Toll-like receptors, including TLR4, play important roles in the early detection of the arrival of pathogenic bacteria in the bladder, initiating pro-inflammatory signaling that culminates in the arrival of neutrophils and other immune cells to the site of infection. Children that present with asymptomatic bacteriuria are more likely to exhibit lower levels of TLR4 on neutrophils than those from control children (144); in addition, these children demonstrate accompanying differences in the expression of certain adaptor proteins in the TLR signaling pathways (144). Polymorphisms in the *Tlr4* promoter region were found to correlate with the magnitude of neutrophil influx into the urinary tract of adults that received experimental UTI with a strain of *E. coli* associated with asymptomatic bacteriuria (145).

### *Interferon regulatory factor 3.*

Irf3 is a cytoplasmic protein that, upon ligation of TLRs or other surface receptors, binds a cytoplasmic partner and translocates into the nucleus, where it upregulates expression of type 1 interferons and other genes. Though this pathway is most tightly linked to immune responses to viral pathogens, mice deficient in production of Irf3 exhibit increased frequency of pyelonephritis, renal abscesses and urosepsis upon transurethral inoculation (146). In humans,

those carrying *Irf3* promoter polymorphisms demonstrated reduced innate immune responses to an asymptomatic bacteriuria strain of *E. coli* (145).

#### *Interleukin-8.*

IL-8, whose gene is a target of TLR- and IL-1-stimulated NF- $\kappa$ B signaling, is a key mediator of neutrophil recruitment in response to bacterial cystitis, and genetic alterations related to IL-8 and its signaling have been linked to susceptibility to pyelonephritis. First, polymorphisms in the IL-8 gene itself were associated with pyelonephritis (147). In addition, low CXCR1 (IL-8 receptor) expression levels resulting from mutations or polymorphisms are correlated with acute and recurrent pyelonephritis (148, 149). Mice deficient in the related chemokine receptor CXCR2 (which recognizes IL-8 and other stimuli) demonstrate increased susceptibility to experimental UTI, renal scarring and urosepsis (150, 151).

#### **Antimicrobial resistance and anti-virulence strategies**

Over the past two decades, the management of extraintestinal *E. coli* and UPEC infections has been complicated by the emergence and expansion of antibiotic resistance (152). Resistance to first-line antibiotics has resulted in delays in appropriate therapy and an increase in morbidity and mortality (153). Indeed, 20-45% of UPEC isolates are now resistant to first-line therapy, according to surveillance studies of Europe and North and South America (154). Particularly alarming is the rapid development of resistance to  $\beta$ -lactam antibiotics among uropathogens due to the emergence of novel  $\beta$ -lactamases, including extended-spectrum  $\beta$ -lactamases (such as CTX-M) (152, 155, 156), metallo- $\beta$ -lactamases (NDM-1) (157-160), and carbapenemase (KPC) (161).

In cystitis, steps inherent to the pathogenicity cascade (*e.g.*, the intracellular location and biofilm-like properties of bacterial communities, and the apparent quiescence of chronically resident reservoir bacteria) may further confound treatment of resistant UTIs, providing organisms with niches where antibiotic penetrance and/or efficacy is insufficient. Novel strategies targeting the mechanisms of pathogenesis may provide an approach that limits antimicrobial resistance by imparting decreased selective pressure on uropathogens. Moreover, such anti-virulence strategies could limit the impact on the microbiota and lessen off-target effects of therapy. Recently, compounds blocking FimH interaction with the bladder epithelium, termed mannosides, prevent UPEC adherence, invasion, and IBC formation in the bladder (162, 163). When dosed orally, mannosides were able to attenuate infection in the murine model of UTI and potentiate efficacy of standard antibiotics, likely by limiting UPEC to a luminal niche where antibiotic concentrations are high (163). Further, compounds inhibiting UPEC pilus biosynthesis (pilicides) by selectively inhibiting either the type P or type 1 chaperones, PapD and FimC, represent another class of rationally designed anti-virulence compounds (164). Pilicide treatment of UPEC abrogates pili formation, without effect on assembled pili, and pretreatment attenuates virulence in mice, suggesting the pilicides may exemplify another promising lead for future anti-virulence therapy (164-166). Additionally, compounds interfering with the regulatory processes governing urovirulence determinants may provide a fertile area for future therapeutics (167, 168). Collectively, these results point to a promising future for development of anti-virulence therapy against UTI.

## Vaccine prospects

With the rise in antibiotic resistance among uropathogens and the frequency with which many women suffer repeated UTIs despite antibiotic treatment, there has also been significant effort invested into the development of vaccines against UPEC. A recent review provides comprehensive documentation of all the evidence-based and clinical investigations that focus on vaccine development against UTIs (169). A notable challenge in the field is that although the goal is to eradicate UPEC from the urinary tract, *E. coli* represents an important component of the gastrointestinal microflora. As such, it would be disadvantageous for any antimicrobial approach to eradicate these commensal forms of *E. coli*. This concern has led investigators to target proteins that are specifically associated with pathogenic strains in an attempt to leave the gastrointestinal flora unperturbed. To date, approaches have included multivalent whole-cell-based cocktails or candidate preparations of individual bacterial components.

### *Individual bacterial components.*

As binding to uroepithelial surfaces is critical for the establishment of UTI, many vaccine candidates are directed against microbial surface structures that would directly or indirectly affect bacterial binding. Uropathogenic strains of *E. coli* are quite heterogeneous in genetic content (51). Surface-expressed molecules that define *E. coli* serotypes might serve as potential immunogens, but these molecules are very heterogeneous among strains isolated from the urinary tract. For example, there are as many as twenty LPS O-antigen serotypes, just one example of the potential diversity in surface molecules among *E. coli* strains that cause UTI. Other classes of vaccine targets that have been investigated include adhesive organelles,



exotoxins, iron receptors, flagella, and capsule (169). These targets have demonstrated efficacy in animal models, but few have been taken forward to any form of clinical trial.

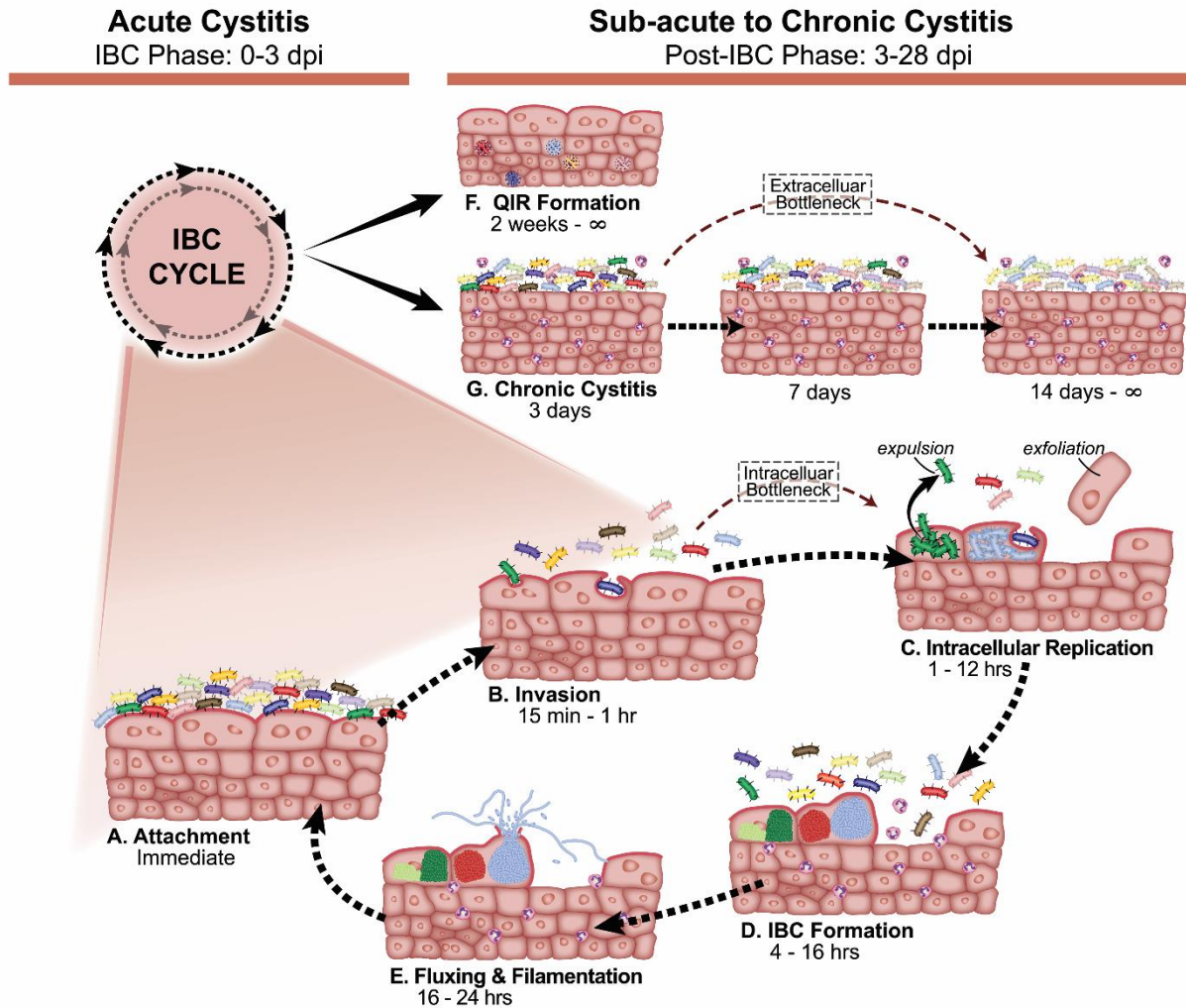
#### *Multivalent approaches.*

These strategies include the use of bacterial proteins in the form of bacterial lysates (Uro-Vaxom®) (170) or intact bacteria (Urovac®, Urvakol®) (171, 172), which offers the advantage of inclusion of a large number of potential immunogens. In fact, numerous *E. coli* serotypes could be included in such a cocktail to broaden potential efficacy against diverse *E. coli* strains. However, there are a number of potential limitations to this approach. First, the growth conditions of the organisms during preparation must approximate those within the urinary tract to encourage production of UTI-relevant molecules. Second, this approach would include molecules that are also common to commensal *E. coli*. Immunostimulation against such common *E. coli* molecules could lead to reduced levels of *E. coli* in the gastrointestinal tract, which could compromise gastrointestinal microbiome integrity, or even elicit unintended gastrointestinal inflammation. Lastly, the presence of a plethora of microbial proteins could reduce the immunogenicity toward any one important target and, as such, the efficacy of vaccination may be adversely affected. Of note, these polyvalent approaches have demonstrated efficacy in the prevention of UTI in animal and human studies (170-172). However, there have been adverse side effects at the delivery site, or a need for repetitive administration in order to attain prolonged efficacy, reducing the enthusiasm for the use of these vaccines.

## Conclusion

Recent years have seen a remarkable increase in our understanding of the virulence attributes and behavior of uropathogenic *E. coli* in the urinary tract. New paradigms raise the possibility that recurrent urinary tract infections may arise via mechanisms other than reinoculation from a gastrointestinal tract reservoir. This new knowledge, as well as the molecular details of pilus assembly, bacterial attachment and epithelial invasion, opens new avenues for development of anti-virulence compounds and strategies. Coupled with renewed interest in vaccine targets for UTI, these approaches are expected to be increasingly important as antimicrobial resistance among UPEC and other uropathogenic bacteria becomes more widespread.

## Figures



**Figure 1. The intracellular bacterial community model of bacterial cystitis.** Infection begins with bacterial attachment to the uroepithelium (**A**), followed shortly by internalization into superficial epithelial cells (**B**). A subset of UPEC gains access to the cytoplasm, replicating into IBCs (**C**, **D**). Ultimately UPEC flux from the infected cell, some organisms adopting a filamentous morphology (**E**) to initiate subsequent rounds of IBC formation. Outcomes of the acute phase of infection include the formation of quiescent intracellular reservoirs (QIRs; **F**) or,

in susceptible hosts, the development of chronic active cystitis (**G**). Reproduced with permission from Reference 105.

**Table 1. Host factors influencing bacterial adherence and invasion**

	Attachment	Invasion	Expulsion / Blocking
Actin		(14, 17)	(14)
cAMP		(11)	(11, 113)
Caveolae		(12)	(113)
Histone deacetylase		(173)	(173)
Integrin	(16)	(16)	
Lipid rafts		(12)	
Microtubules		(173)	(173)
MyRIP			(113)
Rab27b		(11)	(113)
Rho-GTPase		(17)	
TLR4	(113)		(113)
Uroplakin Ia	(26)		
Uroplakin IIIa		(93)	(93)

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APPENDIX TWO

SUBVERSION OF HOST INNATE IMMUNITY BY

UROPATHOGENIC *ESCHERICHIA COLI*

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Modified from Olson and Hunstad (2016) *Pathogens*.

**Abstract**

Uropathogenic *Escherichia coli* (UPEC) cause the majority of community-onset urinary tract infection (UTI) and represent a major etiologic agent of healthcare-associated UTI. Introduction of UPEC into the mammalian urinary tract evokes a well-described inflammatory response, comprising pro-inflammatory cytokines and chemokines as well as cellular elements (neutrophils and macrophages). In human UTI, this inflammatory response contributes to symptomatology and provides means for diagnosis by standard clinical testing. Early in acute cystitis, UPEC gains access to an intracellular niche that protects a population of replicating bacteria from arriving phagocytes. To ensure the establishment of this protected niche, UPEC employ multiple

strategies to attenuate and delay the initiation of host inflammatory components, including epithelial secretion of chemoattractants. Recent work has also revealed novel mechanisms by which UPEC blunts neutrophil migration across infected uroepithelium. Taken together, these attributes distinguish UPEC from commensal and nonpathogenic *E. coli* strains. This review highlights the unique immune evasion and suppression strategies of this bacterial pathogen and offers directions for further study; molecular understanding of these mechanisms will inform the development of adjunctive, anti-virulence therapeutics for UTI.

## **Introduction**

The human immune system faces a constant obligation to provide surveillance at epithelial surfaces and to detect and eliminate pathogenic microbes. Innate responses are triggered by ligation of pathogen-recognition receptors on and within epithelial cells and resident immune cells. These responses activate and recruit phagocytes, particularly macrophages and neutrophils (polymorphonuclear leukocytes; PMNs), which act to engulf and kill bacterial pathogens at the point of their arrival. Conversely, in order to establish infection and thwart immune detection and clearance, microorganisms of all types have evolved a variety of molecular mechanisms to attenuate and evade mammalian innate immunity (reviewed in [1-4] among many others). Even among Gram-negative bacteria, the mechanisms of these effects vary widely. In many model pathogens (*e.g.*, *Salmonella*, *Vibrio*, *Yersinia*), effector proteins interrupting host cell signaling, kinase activation, cytoskeletal rearrangement, post-translational modification, and other processes are delivered to the target cell via type III secretion. Here, we will outline the distinct immune modulation strategies of uropathogenic *Escherichia coli* (UPEC), the chief cause of urinary tract infections [5,6]. Unlike other pathotypes of *E. coli* that

modulate host responses (*e.g.*, enterohemorrhagic or enteropathogenic), UPEC do not encode type III secretion machinery.

The ascending introduction of UPEC or other pathogenic bacteria into the mammalian urinary tract induces an inflammatory response, initiated primarily by activation of Toll-like receptors (TLRs). Stimulation of TLR4 by bacterial lipopolysaccharide (LPS) activates the NF- $\kappa$ B pathway, inducing the expression of cytokines including IL-6 and neutrophil chemoattractants such as IL-8 (CXCL1), which are measurable in the urine of mice and humans with UTI [7,8]. Increases in uroepithelial cyclic AMP, activated by TLR4-induced increases in intracellular  $[Ca^{2+}]$ , also results in NF- $\kappa$ B-independent augmentation of IL-6 and IL-8 expression [9]. TLR5 stimulation, responding to bacterial flagellin, can additionally contribute to acute inflammation during UTI [10,11]. Perpetuation of the neutrophil response may be driven by cytokines such as IL-17, which has an emerging role in bridging innate to adaptive immunity [12] and is a mediator of the innate response during experimental UTI [13]. The human cathelicidin LL-37, a small, cationic antibacterial peptide, is detectable in the urine during human cystitis, and mice deficient in its ortholog (CRAMP) demonstrate increased susceptibility to pyelonephritis [14], although it may paradoxically enhance bladder infection [15]. Multiple members of the defensin class of antimicrobial peptides are also produced locally during UTI, and the participation of these molecules in the host-pathogen conversation remains a fertile area for study.

A core result of the soluble inflammatory response is the recruitment of neutrophils to the bladder. Indeed, the detection of neutrophils in the urine is a diagnostic hallmark of human UTI. The phagocytic capacity of neutrophils plays a critical role in controlling UPEC UTI, as demonstrated in multiple studies [16-21]. UPEC is able to effectively colonize the bladder in the

face of this highly inflammatory soluble and cellular response, first attenuating phagocyte recruitment and subsequently evading the activity of neutrophils in the bladder. The molecular strategies underlying these UPEC phenotypes are the focus of this review.

### **Immune evasion strategies of UPEC**

#### *The UPEC intracellular bacterial community.*

As detailed elsewhere in this issue, acute UPEC cystitis relies on a well-documented intracellular pathogenesis cascade in which the pathogen is internalized into superficial bladder epithelial cells (also termed facet or umbrella cells) [22-33]. Despite comprising a minority of total bacteria within the infected bladder (most are luminal), intracellular UPEC coordinate a variety of essential functions to propagate infection. The facet cell cytoplasm provides a rich nutrient environment that supports the massive intracellular replication of UPEC in the acute phase of cystitis, forming the intracellular bacterial community (IBC) [28]. However, this locale also represents a vitally important, protected niche whereby UPEC can evade the phagocytic activity of arriving neutrophils. By video microscopy in the infected murine bladder, neutrophils can be observed engulfing luminal bacilli during the initial hours of infection [34]. However, bacteria that have successfully invaded uroepithelial cells are sheltered from this attack; neutrophils are clearly able to locate infected epithelial cells, but cannot access the expanding bacterial population comprising the IBC (**Figure 1**). As part of the host response to infection, many of the facet cells will ultimately be exfoliated into the urine – a means by which the host may rid itself of many thousands of bacteria. In order to escape this fate and perpetuate infection, a subset of UPEC must exit the IBC and initiate additional genetic programs that allow



these bacteria to traverse the extracellular space in the face of a burgeoning luminal neutrophil population, thereby facilitating additional rounds of IBC formation in naïve epithelial cells.

### *Filamentation.*

During maturation of the IBC, a minority of the intracellular population proceeds through a differentiation pathway that results in the formation of non-septated, filamentous bacteria up to 70  $\mu\text{m}$  in length – a process that requires expression of the cell division inhibitor *SulA* [34,35]. Upon lysis of the IBC-harboring facet cell, UPEC flux out of the IBC proper to traverse the luminal surface. Video microscopy demonstrates that during this time of transition, bacillary forms of UPEC (1-2  $\mu\text{m}$  in length) are readily engulfed, while filamentous UPEC resist phagocytosis, even when in direct contact with PMNs and macrophages [35,36] (**Figure 2**). At this juncture during murine cystitis, flow cytometric analysis of bladder luminal bacteria demonstrates enrichment of filamentous forms, consistent with a selection process in which bacillary forms are preferentially taken up by host phagocytes [34]. This preferential engulfment of standard bacilli is also supported by in vitro studies using cultured bladder epithelial cells and human macrophages [34,36]. As PMNs are in fact capable of engulfing particles (e.g., fungal hyphae) that are larger than themselves [37,38], the observed resistance of filamentous UPEC to PMN phagocytosis likely relies on attributes beyond the organism's size [35]. Underscoring the pathogenic importance of filamentation, the UPEC *sulA* mutant forms a first round of IBCs without difficulty, but fails to sustain infection beyond this point, as it cannot filament in order to survive the luminal transition. Abrogation of the PMN response (e.g., in TLR4-deficient mice) rescues the *sulA* pathogenic defect [35].

The adoption of the filamentous phenotype by a subset of UPEC represents a response of the pathogen to stresses associated with the marshaling of host immune components. In support of this model, filamentation is not observed during UPEC UTI in TLR4-deficient hosts, which feature a sharply limited soluble and cellular inflammatory response to bacterial introduction. Meanwhile, filamentation can be induced *in vitro* by exposure of bacillary UPEC to LPS-activated macrophages [34,36]. Taken together, these observations demonstrate an elegant host-pathogen conversation taking place during this critical stage of UTI propagation.

#### *UPEC attenuation of early uroepithelial cytokine production.*

While the full extent of the neutrophilic response to bacterial cystitis is fully evident within hours, internalization of UPEC into bladder epithelial cells occurs initially in < 1 h [28,39]. Substantial work by multiple groups has outlined a number of pathogenic strategies by which UPEC may dampen very early innate responses, effectively “holding off the cavalry” to protect luminal bacteria until the protected intracellular niche can be established.

Compared with commensal or laboratory strains of *E. coli*, UPEC display a marked ability to dominantly suppress epithelial cytokine production. UPEC stabilize I $\kappa$ B, thereby suppressing NF- $\kappa$ B activity and increasing bladder epithelial cell apoptosis [40]. Further, multiple UPEC isolates elicit lower levels of IL-6 and IL-8 from uroepithelial cells and are able to block secretion of these inflammatory cytokines upon co-inoculation with known NF- $\kappa$ B stimulators [41,42]. Genes involved in LPS biosynthesis (*e.g.*, *rfa*, *rfb*, *waaL*) are also important for this phenotype, suggesting that UPEC LPS modifications may at least confer a less stimulatory LPS structure, or perhaps that a non-stimulatory LPS may exert a dominant effect [42,43]. In addition, lack of the OMP chaperone SurA abolishes the immunosuppressive phenotype [42];

reduced levels of the SurA-dependent LPS transport protein LptD may alter the presentation of potentially non-stimulatory LPS [44].

However, LPS differences alone do not account for cytokine suppression [45], and it is likely that other mechanisms and UPEC effectors also contribute to suppressing cytokine secretion. For example, NF- $\kappa$ B signaling may be inhibited by sub-lytic concentrations of  $\alpha$ -hemolysin (HlyA) [46,47], which is encoded on a UPEC pathogenicity island that also harbors *CnfI* (see section 2.4). Exemplifying a distinct strategy, a substantial minority of UPEC isolates, including the pyelonephritis/urosepsis strain CFT073, encode a Toll/IL-1 receptor (TIR) domain-containing protein, termed TcpC, that interacts with the host adaptor MyD88 to inhibit TLR signaling [48]. TcpC disruption was associated with decreased bacterial burden in the kidneys and a reduction in histologically evident renal damage, indicating the importance of this protein during pyelonephritis. Further study of additional signaling pathways that rely on TIR domain-containing proteins (*e.g.*, the TRIF and IL-6/IL-1 signaling cascades) revealed that TcpC also regulated these pathways independent of MyD88, suggesting that TcpC may impact pathogenesis in a broader way [49]. Finally, the SisA and SisB proteins, homologues of the immunomodulatory protein ShiA from *Shigella flexneri*, are found in CFT073 and have recently been implicated in immune modulation during UTI [50].

#### *UPEC inhibition of neutrophil recruitment and function*

Uropathogenic strains of *E. coli* feature additional virulence mechanisms targeting the recruitment and function of neutrophils. In contrast to commensal and laboratory *E. coli* isolates, UPEC are able to suppress neutrophil migration by down-regulating expression of many PMN genes involved in neutrophil chemotaxis, proinflammatory signaling, adhesion, and migration

[51]. To study the molecular determinants of this phenomenon, we recently optimized an *in vitro* model of PMN migration across a cultured bladder epithelial cell monolayer [51-53].

UPEC isolates both attenuate transepithelial PMN migration *in vitro* and elicit reduced bladder PMN influx *in vivo*, in contrast to nonpathogenic strains [51,53].

UPEC have been shown to induce a number of anti-inflammatory molecules within PMNs and uroepithelial cells, including indoleamine 2,3-dioxygenase (IDO) [51]. IDO represents the first enzyme in the catabolism of tryptophan and is well known to modulate adaptive immunity, specifically T cell functions, by starving the local milieu of tryptophan [54]. Unlike nonpathogenic *E. coli*, UPEC specifically upregulate IDO locally within 1 h after introduction into the murine bladder, resulting in attenuation of innate cellular responses (i.e., neutrophil recruitment). Compared with wild-type hosts, UTI in IDO-deficient mice features augmented PMN migration, corresponding with increased killing of extracellular (luminal) bacteria and attenuation of infection [55]. Though the key IDO-expressing cell types have not yet been specified, contributions from both the epithelial and hematopoietic compartments are being investigated. Independent of this question, the sequential influence of two canonical IDO stimulators, namely TNF $\alpha$  and interferons (both type I and IFN $\gamma$ ), appears to underlie UPEC induction of IDO in the host ([55] and unpublished data). Notably, IDO influence on PMN migration is not mediated via tryptophan starvation, as experimental addition of exogenous tryptophan does not rescue neutrophil migration. Instead, the products of tryptophan catabolism, known collectively as kynurenines, may exert a direct influence on PMN transuroepithelial migration.

UPEC also employ a variety of secreted toxins and effectors to neutralize the phagocytic activity of PMNs. As noted above,  $\alpha$ -hemolysin (HlyA) is cytolytic to hematopoietic cells but

also has subtler immune-modulating effects at sub-lytic concentrations [56,57]. The genetically linked UPEC toxin termed cytotoxic necrotizing factor 1 (CNF1) suppresses PMN chemotaxis, phagocytic activity and release of reactive oxygen species [58,59]. Interestingly, CNF1 appears to be packaged into outer membrane vesicles, structures liberated by all Gram-negative bacteria, for delivery to the target phagocyte [59]. A newly identified UPEC effector, termed YbcL, has been shown to inhibit transepithelial migration of human neutrophils *in vivo* and *in vitro* via a mechanism that is distinct but as yet incompletely defined. This effector localizes initially to the bacterial periplasm and is subsequently liberated via bacterial lysis, suppressing PMN transepithelial migration in both *in vivo* and *in vitro* model systems [53,60]. YbcL homologs are present in laboratory and commensal *E. coli* isolates but are unable to suppress PMN migration due to a single amino acid alteration, while most UPEC strains encode the suppressive YbcL variant [53]. Interestingly, YbcL suppression of PMN migration requires the presence of uroepithelial cells (i.e., it does not act directly on PMN alone), and the bacterial lysis that liberates YbcL is augmented by bacterial exposure to bladder epithelia [60]. These observations highlight a form of bacterial altruism in which a subset of UPEC arriving in the mammalian bladder are sacrificed such that others in the nascent UPEC community may achieve success in colonizing the bladder epithelium.

### **Does inflammation ultimately *promote* UPEC infection?**

A recent body of literature supports a model in which host inflammation during UTI may actually represent a double-edged sword. As has been discussed, the acute inflammatory response is essential for controlling UTI [17,61,62]; however, overly exuberant inflammatory responses during acute cystitis may be associated with increased tissue damage, predisposing the

host to developing chronic forms of infection [63,64]. In susceptible murine hosts (*e.g.*, C3H/HeN), a fraction of mice are unable to resolve acute UTI, developing long-term chronic bacterial cystitis with ongoing high tissue bacterial burden and inflammation. This appears to arise as the result of an early immune checkpoint, which can be altered experimentally by prior treatment with anti-inflammatory agents, subsequently preventing chronic infection [63,65]. Further, after chronic cystitis has become established, hosts are unable to resolve infection despite an intense, ongoing leukocyte response, and restoration of the exfoliated transitional epithelium is inhibited [63]. This inflamed state may thus allow UPEC access to more protected niches and deeper cell layers. Thus, hosts need to mount a finely tuned inflammatory response in order to eliminate uropathogens while avoiding the detrimental consequences of an exaggerated response that may augment disease severity or promote persistent infection.

## **Conclusions and Future Directions**

Our knowledge of acute immunity during UTI and the mechanisms that UPEC exploit to evade it has increased dramatically over the past decade. Ongoing work aims to establish the molecular mechanisms of recently published immune evasion effectors and strategies. For example, previous studies suggest that YbcL has greatest structural homology to the mammalian Raf kinase inhibitory protein and may therefore interact with eukaryotic kinases [66]; specifying putative binding partners and targets within a host cell may elucidate the mechanism of this novel PMN trafficking inhibitor. In the same light, we are investigating how kynurenines produced by IDO tryptophan catabolism influence neutrophil migration. These studies will further illuminate the complex host-pathogen interactions occurring in the very early stages of UTI establishment.

Similarly, our understanding of protective immunity and the predictors of recurrent UTI is incomplete. Multiple groups are continuing work on the humoral immune response to cystitis and pyelonephritis and identification of immunodominant antigens. As well, studies of adaptive cellular responses with correlation to recurrence risk should be prioritized. In the decade to come, it is hoped that such investigations will enable novel approaches to limit the morbidity of these recalcitrant infections.

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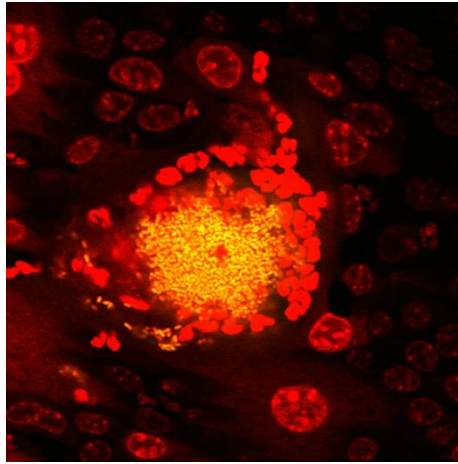
### **Author Contributions**

P.D.O and D.A.H. wrote the manuscript.

### **Conflict of Interest**

D.A.H. serves as a Scientific Advisor for BioVersys AG, Basel, Switzerland.

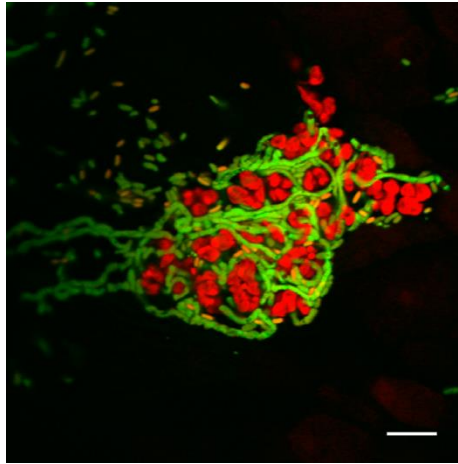
## Figures



**Figure 1. Intracellular UPEC communities are protected from neutrophil attack.**

Confocal microscopy during acute murine cystitis, viewed from a luminal perspective, shows an intracellular bacterial community of UPEC (yellow) within a binucleate superficial facet cell that is surrounded by recruited neutrophils (revealed by red nuclear staining; larger, round epithelial cell nuclei are also visible).





**Figure 2. Filamentous UPEC resist phagocytosis in the bladder.** Confocal microscopy shows a (luminal) surface collection of bacillary and filamentous UPEC (green) in which the bacterial filaments are heavily intermingled with neutrophils (red nuclei). Scale bar, 10  $\mu\text{m}$ .

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